

*Application
for
United States Letters Patent*

To all whom it may concern:

Be it known that

Ron S. Israeli, Warren D. W. Heston, William R. Fair, Ouathek
Ouerfelli and John Pinto

have invented certain new and useful improvements in

PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

of which the following is a full, clear and exact description.

PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

5

10

This invention disclosed herein was made in part with Government support under Grants Nos. DK47650 and
15 CA58192, CA-39203, CA-29502, CA-08748-29 from the National Institute of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

20 BACKGROUND OF THE INVENTION

Throughout this application various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby
25 incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each set of Examples in the Experimental Details section.

30

Prostate cancer is among the most significant medical problems in the United States, as the disease is now the most common malignancy diagnosed in American males. In 1992 there were over 132,000 new cases of prostate
35 cancer detected with over 36,000 deaths attributable to the disease, representing a 17.3% increase over 4 years (8). Five year survival rates for patients with prostate cancer range from 88% for those with localized disease to 29% for those with metastatic disease. The

rapid increase in the number of cases appears to result in part from an increase in disease awareness as well as the widespread use of clinical markers such as the secreted proteins prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) (7).

The prostate gland is a site of significant pathology affected by conditions such as benign growth (BPH), neoplasia (prostatic cancer) and infection (prostatitis). Prostate cancer represents the second leading cause of death from cancer in man (7). However prostatic cancer is the leading site for cancer development in men. The difference between these two facts relates to prostatic cancer occurring with increasing frequency as men age, especially in the ages beyond 60 at a time when death from other factors often intervenes. Also, the spectrum of biologic aggressiveness of prostatic cancer is great, so that in some men following detection the tumor remains a latent histologic tumor and does not become clinically significant, whereas in other it progresses rapidly, metastasizes and kills the man in a relatively short 2-5 year period (7 and 59).

In prostate cancer cells, two specific proteins that are made in very high concentrations are prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) (21, 47, and 65). These proteins have been characterized and have been used to follow response to therapy. With the development of cancer, the normal architecture of the gland becomes altered, including loss of the normal duct structure for the removal of secretions and thus the secretions reach the serum. Indeed measurement of serum PSA is suggested as a potential screening method for prostatic cancer. Indeed, the relative amount of PSA and/or PAP in the cancer reduces as compared to normal or benign tissue.

PAP was one of the earliest serum markers for detecting metastatic spread (47). PAP hydrolyses tyrosine phosphate and has a broad substrate specificity. Tyrosine phosphorylation is often increased with
5 oncogenic transformation. It has been hypothesized that during neoplastic transformation there is less phosphatase activity available to inactivate proteins that are activated by phosphorylation on tyrosine residues. In some instances, insertion of phosphatases
10 that have tyrosine phosphatase activity has reversed the malignant phenotype.

PSA is a protease and it is not readily appreciated how loss of its activity correlates with cancer development
15 (21, and 65). The proteolytic activity of PSA is inhibited by zinc. Zinc concentrations are high in the normal prostate and reduced in prostatic cancer. Possibly the loss of zinc allows for increased proteolytic activity by PSA. As proteases are involved
20 in metastasis and some proteases stimulate mitotic activity, the potentially increased activity of PSA could be hypothesized to play a role in the tumors metastases and spread (39).

25 Both PSA and PAP are found in prostatic secretions. Both appear to be dependent on the presence of androgens for their production and are substantially reduced following androgen deprivation.

30 Prostate-specific membrane antigen (PSM) which appears to be localized to the prostatic membrane has been identified. This antigen was identified as the result of generating monoclonal antibodies to a prostatic cancer cell, LNCaP (22).

35

Dr. Horoszewicz established a cell line designated LNCaP from the lymph node of a hormone refractory,

heavily pretreated patient (23). This line was found to have an aneuploid human male karyotype. It maintained prostatic differentiation functionality in that it produced both PSA and PAP. It possessed an
5 androgen receptor of high affinity and specificity. Mice were immunized with LNCaP cells and hybridomas were derived from sensitized animals. A monoclonal antibody was derived and was designated 7E11-C5 (22). The antibody staining was consistent with a membrane
10 location and isolated fractions of LNCaP cell membranes exhibited a strongly positive reaction with immunoblotting and ELISA techniques. This antibody did not inhibit or enhance the growth of LNCaP cells in vitro or in vivo. The antibody to this antigen was
15 remarkably specific to prostatic epithelial cells, as no reactivity was observed in any other component. Immunohistochemical staining of cancerous epithelial cells was more intense than that of normal or benign epithelial cells.

20 Dr. Horoszewicz also reported detection of immunoreactive material using 7E11-C5 in serum of prostatic cancer patients (22). The immunoreactivity was detectable in nearly 60% of patients with stage D-2
25 disease and in a slightly lower percentage of patients with earlier stage disease, but the numbers of patients in the latter group are small. Patients with benign prostatic hyperplasia (BPH) were negative. Patients with no apparent disease were negative, but 50-60% of
30 patients in remission yet with active stable disease or with progression demonstrated positive serum reactivity. Patients with non prostatic tumors did not show immunoreactivity with 7E11-C5.

35 The 7E11-C5 monoclonal antibody is currently in clinical trials. The aldehyde groups of the antibody were oxidized and the linker-chelator glycol-tyrosyl-

(n, ε-diethylenetriamine-pentacetic acid)-lysine (GYK-DTPA) was coupled to the reactive aldehydes of the heavy chain. The resulting antibody was designated CYT-356. Immunohistochemical staining patterns were similar except that the CYT-356 modified antibody stained skeletal muscle. The comparison of CYT-356 with 7E11-C5 monoclonal antibody suggested both had binding to type 2 muscle fibers. The reason for the discrepancy with the earlier study, which reported skeletal muscle to be negative, was suggested to be due to differences in tissue fixation techniques. Still, the most intense and definite reaction was observed with prostatic epithelial cells, especially cancerous cells. Reactivity with mouse skeletal muscle was detected with immunohistochemistry but not in imaging studies. The Indium¹¹¹-labeled antibody localized to LNCaP tumors grown in nude mice with an uptake of nearly 30% of the injected dose per gram tumor at four days. In-vivo, no selective retention of the antibody was observed in antigen negative tumors such as PC-3 and DU-145, or by skeletal muscle. Very little was known about the PSM antigen. An effort at purification and characterization has been described at meetings by Dr. George Wright and colleagues (14 and 64).

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1C:

5 Immunohistochemical detection of PSM
antigen expression in prostate cell
lines. Top panel reveals uniformly
high level of expression in LNCaP
cells; middle panel and lower panel are
10 DU-145 and PC-3 cells respectively,
both negative.

Figure 2: Autoradiogram of protein gel revealing
products of PSM coupled in-vitro
transcription/translation. Non-
15 glycosylated PSM polypeptide is seen at
84 kDa (lane 1) and PSM glycoprotein
synthesized following the addition of
microsomes is seen at 100 kDa (lane 2).

20 Figure 3: Western Blot analysis detecting PSM
expression in transfected non-PSM
expressing PC-3 cells. 100 kDa PSM
glycoprotein species is clearly seen in
LNCaP membranes (lane 1), LNCaP crude
25 lysate (lane 2), and PSM-transfected
PC-3 cells (lane 4), but is
undetectable in native PC-3 cells (lane
3).

30 Figure 4: Autoradiogram of ribonuclease
protection gel assaying for PSM mRNA
expression in normal human tissues.
Radiolabeled 1 kb DNA ladder (Gibco-
BRL) is shown in lane 1. Undigested
35 probe is 400 nucleotides (lane 2),
expected protected PSM band is 350
nucleotides, and tRNA control is shown

5 (lane 3). A strong signal is seen in
human prostate (lane 11), with very
faint, but detectable signals seen in
human brain (lane 4) and human salivary
gland (lane 12). No signal was detected
in lane 5 kidney, lane 6 liver, lane 7
lung, lane 8 mammary gland, lane 9
pancreas, lane 10 placenta, lane 13
skeletal muscle, lane 14 spleen, and
10 lane 15 testes.

Figure 5: Autoradiogram of ribonuclease
protection gel assaying for PSM mRNA
expression in LNCaP tumors grown in
15 nude mice, and in human prostatic
tissues. ³²P-labeled 1 kb DNA ladder is
shown in lane 1. 298 nucleotide
undigested probe is shown (lane 2), and
tRNA control is shown (lane 3). PSM
20 mRNA expression is clearly detectable
in LNCaP cells (lane 4), orthotopically
grown LNCaP tumors in nude mice with
and without matrigel (lanes 5 and 6),
and subcutaneously implanted and grown
25 LNCaP tumors in nude mice (lane 7).
PSM mRNA expression is also seen in
normal human prostate (lane 8), and in
a moderately differentiated human
prostatic adenocarcinoma (lane 10).
30 Very faint expression is seen in a
sample of human prostate tissue with
benign hyperplasia (lane 9).

Figure 6: Ribonuclease protection assay for PSM
35 expression in LNCaP cells treated with
physiologic doses of various steroids
for 24 hours. ³²P-labeled DNA ladder is

5 shown in lane 1. 298 nucleotide
undigested probe is shown (lane 2), and
tRNA control is shown (lane 3). PSM
mRNA expression is highest in untreated
LNCaP cells in charcoal-stripped media
(lane 4). Applicant see significantly
diminished PSM expression in LNCaP
cells treated with DHT (lane 5),
Testosterone (lane 6), Estradiol (lane
10 7), and Progesterone (lane 8), with
little response to Dexamethasone (lane
9).

15 **Figure 7:** Data illustrating results of PSM DNA
and RNA presence in transfect Dunning
cell lines employing Southern and
Northern blotting techniques

20 **Figures 8A-8B:**
Figure A indicates the power of
cytokine transfected cells to teach
unmodified cells. Administration was
directed to the parental flank or
prostate cells. The results indicate
25 the microenvironment considerations.

30 Figure B indicates actual potency at a
particular site. The tumor was
implanted in prostate cells and treated
with immune cells at two different
sites.

Figures 9A-9B:
35 Relates potency of cytokines in
inhibiting growth of primary tumors.
Animals administered un-modified
parental tumor cells and administered

as a vaccine transfected cells.
Following prostatectomy of rodent tumor
results in survival increase.

5 **Figure 10:** PCR amplification with nested primers
improved the level of detection of
prostatic cells from approximately one
prostatic cell per 10,000 MCF-7 cells
to better than one cell per million
10 MCF-7 cells, using PSA.

Figure 11: PCR amplification with nested primers
improved the level of detection of
prostatic cells from approximately one
15 prostatic cell per 10,000 MCF-7 cells
to better than one cell per million
MCF-7 cells, using PSM-derived primers.

Figure 12: A representative ethidium stained gel
20 photograph for PSM-PCR. Samples run in
lane A represent PCR products generated
from the outer primers and samples in
lanes labeled B are products of inner
primer pairs.

25 **Figure 13:** PSM Southern blot autoradiograph. The
sensitivity of the Southern blot
analysis exceeded that of ethidium
staining, as can be seen in several
30 samples where the outer product is not
visible, but is detectable by Southern
blotting.

Figure 14: Characteristics of the 16 patients
35 analyzed with respect to their clinical
stage, treatment, serum PSA and PAP
values, and results of assay.

Figures 15A-15D:

5 DNA sequence containing promoter elements from nucleotide -1 to nucleotide -3017. -1 is upstream of start site of PSM.

Figure 16: Potential binding sites on the PSM promoter fragment.

10 Figure 17: Promoter activity of PSM up-stream fragment/CAT gene chimera.

15 Figure 18: Comparison between PSM and PSM' cDNA. Sequence of the 5' end of PSM cDNA (32) is shown. Underlined region (beginning at nucleotide 115 and continuing to nucleotide 380) denotes nucleotides which are absent in PSM' cDNA but present in PSM cDNA.. Boxed region represents the putative transmembrane domain of PSM antigen. * Asterisk denotes the putative translation initiation site for PSM'.

20

25 Figure 19: Graphical representation of PSM and PSM' cDNA sequences and antisense PSM RNA probe (b). PSM cDNA sequence with complete coding region (32). (a) PSM' cDNA sequence from this study. (c) Cross hatched and open boxes denote sequences identity in PSM and PSM'. Hatched box indicates sequence absent from PSM'. Regions of cDNA sequence complementary to the antisense probe are indicated by dashed lines between the sequences.

30

35

Figure 20: RNase protection assay with PSM specific probe in primary prostatic tissues. Total cellular RNA was isolated from human prostatic samples: normal prostate, BPH, and CaP. PSM and PSM' spliced variants are indicated with arrows at right. The left lane is a DNA ladder. Samples from different patients are classified as: lanes 3-6, CaP, carcinoma of prostate; BPH, benign prostatic hypertrophy, lanes 7-9; normal, normal prostatic tissue, lanes 10-12. Autoradiograph was exposed for longer period to read lanes 5 and 9.

Figure 21: Tumor Index, a quantification of the expression of PSM and PSM'. Expression of PSM and PSM' was quantified by densitometry and expressed as a ratio of PSM/PSM' on the Y-axis. Three samples each were quantitated for primary CaP, BPH and normal prostate tissues. Two samples were quantitated for LNCaP. Normal, normal prostate tissue.

Figure 22: Characterization of PSM membrane bound and PSM' in the cytosol.

Figure 23: Photograph of ethidium bromide stained gel depicting representative negative and positive controls used in the study. Samples 1-5 were from, respectively: male with prostatitis, a healthy female volunteer, a male with BPH, a control 1:1,000,000 dilution of LNCaP cells, and a patient with renal

cell carcinoma. Below each reaction is the corresponding control reaction performed with beta-2-microglobulin primers to assure RNA integrity. No PCR products were detected for any of these negative controls.

5

Figure 24:

10

15

20

Photograph of gel displaying representative positive PCR results using PSM primers in selected patients with either localized or disseminated prostate cancer. Sample 1-5 were from, respectively: a patient with clinically localized stage T₁ disease, a radical prostatectomy patient with organ confined disease and a negative serum PSA, a radical prostatectomy patient with locally advanced disease and a negative serum PSA, a patient with treated stage D2 disease, and a patient with treated hormone refractory disease.

Figure 25:

25

Chromosomal location of PSM based on in-situ hybridization with cDNA and with genomic cosmids.

Figure 26:

30

35

Human monochromosomal somatic cell hybrid blot showing that chromosome 11 contained the PSM genetic sequence by Southern analysis. DNA panel digested with PstI restriction enzyme and probed with PSM cDNA. Lanes M and H refer to mouse and hamster DNAs. The numbers correspond to the human chromosomal DNA in that hybrid.

- 5 **Figure 27:** Ribonuclease protection assay using PSM
radiolabeled RNA probe reveals an
abundant PSM mRNA expression in AT6.1-
11 clone 1, but not in AT6.1-11 clone
2, thereby mapping PSM to 11p11.2-13
region.
- 10 **Figure 28:** Tissue specific expression of PSM RNA
by Northern blotting and RNase
protection assay.
- 15 **Figure 29:** Mapping of the PSM gene to the 11p11.2-
p13 region of human chromosome 11 by
southern blotting and in-situ
hybridization.
- 20 **Figure 30:** Schematic of potential response
elements.
- 25 **Figure 31:** Schematic depiction of metastatic
prostate cell transfected with promoter
for PSM which is driving expression of
prodrug activating enzyme cytosine
deaminase. This allows for prostate
specific expression and tumor localized
conversion of non-toxic 5
fluorocytosine to 5 flurouracil.
- 30 **Figure 32A-32C:**
Nucleic acid of PSM genomic DNA is read
5 prime away from the transcription
start site: number on the sequences
indicates nucleotide upstream from the
start site. Therefore, nucleotide #121
35 is actually -121 using conventional
numbering system.

- 5 **Figure 33:** Representation of NAAG 1, acivudin, azotomycin, and 6-diazo-5-oxo-norleucine, DON.
- 10 **Figure 34:** Representation of N-acetylaspartylglutamate (NAAG), PALA, PALAGLU, phosphonate antagonist of glutamate receptor and phosphonates of PALAGLU and NAAG.
- 15 **Figure 35:** Synthesis of N-acetylaspartylglutamate, NAAG 1.
- 20 **Figure 36:** Synthesis of N-phosphonoacetylaspartyl-L-glutamate.
- 25 **Figure 37:** Synthesis of 5-diethylphosphonon-2 amino benzylvalerate intermediate.
- 30 **Figure 38:** Synthesis of analog 4 and 5.
- 35 **Figure 39:** Representation of DON, analogs 17-20.
- Figure 40:** Substrates for targeted drug delivery, analog 21 and 22.
- Figure 41:** Dynemycin A and its mode of action.
- Figure 42:** Synthesis of analog 28.
- Figure 43:** Synthesis for intermediate analog 28.
- Figure 44:** Attachment points for PALA.
- Figure 45:** Mode of action for substrate 21.

Figures 46A-46D:

Intron 1F: Forward Sequence.

Figures 47A-47E:

5 Intron 1R: Reverse Sequence

Figures 48A-48C:

Intron 2F: Forward Sequence

10 **Figures 49A-49C:**

Intron 2R: Reverse Sequence

Figures 50A-50B:

15 Intron 3F: Forward Sequence

Figures 51A-51B:

Intron 3R: Reverse Sequence

20 **Figures 52A-52C:**

Intron 4F: Forward Sequence

Figures 53A-53E:

25 Intron 4RF: Reverse Sequence

Figure 54: PSM genomic organization of the exon and 19 intron junction sequences. The exon/intron junctions are as follows:

- 30
1. Exon /intron 1 at bp 389-390;
 2. Exon /intron 2 at bp 490-491;
 3. Exon /intron 3 at bp 681-682;
 4. Exon /intron 4 at bp 784-785;
 5. Exon /intron 5 at bp 911-912;
 6. Exon /intron 6 at bp 1096-1097;

35

 7. Exon /intron 7 at bp 1190-1191;
 8. Exon /intron 8 at bp 1289- 1290;
 9. Exon /intron 9 at bp 1375-1376;

10. Exon /intron 10 at bp 1496-1497;
11. Exon /intron 11 at bp 1579-1580;
12. Exon /intron 12 at bp 1643-1644;
13. Exon /intron 13 at bp 1710-1711;
5 14. Exon /intron 14 at bp 1803-1804;
15. Exon /intron 15 at bp 1894-1895;
16. Exon /intron 16 at bp 2158-2159;
17. Exon /intron 17 at bp 2240-2241;
18. Exon /intron 18 at bp 2334-2335;
10 19. Exon /intron 19 at bp 2644-2645.

Figures 55A-55J:

Alternatively spliced PSM (PSM') nucleic acid sequence and amino acid sequence.

15

Figure 56:

PSM pteroyl (folate) hydrolase activity in LNCaP membrane preparation. Time course of MTXglu₃ hydrolysis (- ■ -) and concurrent formation of MTXglu₂ (- -), MTXglu₁ (- ▲ -), and MTX (- -), respectively. Membrane fractions were prepared as described in Methods. Reaction volume was 100 µL containing 50 mM acetate/Triton buffer pH 4.5, 50 µM MTXglu₃, 10 µg/mL protein. Values are $\bar{x} \pm$ S.D. from three separate LNCaP membrane preparations.

20

25

Figure 57:

PSM pteroyl (folate) hydrolase activity of immunoprecipitated PSM antigen. Diagram shows typical capillary electrophoretic separation patterns of MTXglu_(n) derivatives at 0, 30, 60 and 240 minute reaction times. Elution intervals for MTXglu₃, MTXglu₂, MTXglu₁, and MTX are 4.25, 3.95, 3.55, and 3.06 min, respectively. Total volume of

30

35

reaction mixture was 100 uL containing 50 uM MTXglu₃.

Figure 58:

5 Effects of pH on gamma-glutamyl
hydrolase (PSM hydrolase) activity in
LNCaP, PC-3 PSM-transfected (PC-3(+))
and PSM non-transfected (PC-3(-))
cells. Enzymic activity is reported as
10 μ M MTXglu₂ formed/mg protein. Each
value represents the mean of 3
reactions containing 50-60 μ g/mL
protein. The following buffers were
used in 50 mM concentrations spanning a
pH range of 2 to 10: glycine-HCl, pH
15 2.2 to 3.6; acetate, pH 3.6 to 5.6; 2-
(N-morpholino)ethanesulfonic acid
(MES), pH 5.6 to 6.8;
Tris(hydroxymethyl)aminomethane (TRIS),
pH 7 to 8.5; and glycine-NaOH, pH 8.6
20 to 10.0.

Figure 59:

Comparison of pteroyl hydrolase
activity in membranes isolated from
LNCaP, PC-3, TSU-Pr1, and Duke-145
25 adenocarcinoma cell lines. Membranes
were isolated as described in Methods.
Each value represents the mean of
triplicate reactions normalized to 1
mg/mL protein.

Figure 60A-60C:

Immunohistochemical analysis of LNCaP
and PC-3 PSM-transfected and PSM-non-
transfected cells. A 2.65 kb PSM cDNA
containing a hygromycin selection
35 vector was cloned into non PSM-antigen
expressing PC-3 cells and maintained in

regular media supplemented with
hygromycin B. As a control, PC-3 cells
were also transfected with the pREP7
vector alone (PC-3 PSM non-transfected
5 cells). Cells were permeabilized in
acetone/methanol (1:1 v/v) mixture,
blocked with 5% bovine serum
albumin/Tris buffered saline (TBS) and
the 7E11-C5 monoclonal PSM antibody was
10 added to cells. A secondary anti-mouse
IgG₁ antibody conjugated with alkaline
phosphatase was added and PSM-positive
cell staining performed with
bromochloroindolylphenol phosphate.
15 Panel A demonstrates intense
immunoreactivity associated with LNCaP
cells using the monoclonal PSM
antibody; In panel B, comparable
staining occurs in PC-3 cells
20 transfected with PSM expression vector.
Panel C illustrates PC-3 cells
expressing pREP7 hygromycin vector
alone.

25 **Figure 61:** Comparison of pteroyl (folate)
hydrolase activity in membranes
isolated from PSM expressing PC-3 cells
and PC-3 cells expressing pREP7
hygromycin vector alone. Membranes were
30 isolated as described in Methods. Each
value represents the mean of triplicate
reactions normalized to 1 mg/mL
protein.

35 **Figure 62:** Representation of N-
acetylaspartylglutamate (NAAG), folic
acid, folate-gamma-polyglutamate,

methotrexate, methotrexate-gamma-
polyglutamate, methotrexate-alpha-
monoglutamate, methotrexate-gamma-
diglutamate, methotrexate-gamma-
5 triglutamate, methotrexate-gamma-
tétraglutamate.

Figure 63A-63B:

10 Solid phase synthesis of methotrexate
alpha-polyglutamatae analogs.

Figure 64: Sequence analysis of microsatellite
instability in PSM gene.

15 **Figure 65:** PSM genomic organization.

Figure 66: Location of microsatellite in PSM gene

SUMMARY OF THE INVENTION

5 This invention provides an isolated nucleic acid molecule encoding an alternatively spliced human prostate-specific membrane antigen. This invention provides an isolated nucleic acid comprising a promoter sequence normally associated with the transcription of a gene encoding a human prostate-specific membrane antigen. This invention provides an isolated
10 polypeptide having the biological activity of an alternatively spliced prostate-specific membrane antigen.

15 This invention provides a method of detecting a nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen and a method of detecting a prostate tumor cell in a subject.

20 Lastly, this invention provides a pharmaceutical composition comprising a compound in a therapeutically effective amount and a pharmaceutically acceptable carrier and a method of making prostate cells susceptible to a cytotoxic agent.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides an isolated nucleic acid encoding an alternatively spliced human prostate-specific membrane (PSM') antigen. As defined herein
5 "nucleic acid encoding an alternatively spliced prostate-specific membrane (PSM') antigen" means nucleic acid encoding a prostate-specific membrane antigen which contains a deletion in the DNA sequence
10 encoding prostate specific membrane antigen between nucleotide 115 and 380. In one embodiment the isolated nucleic acid encodes the alternatively spliced human prostate-specific membrane antigen as set forth in Figure 55.

15 This invention further provides an isolated mammalian genomic DNA molecule which encodes an alternatively spliced prostate-specific membrane antigen. This invention further provides an isolated mammalian DNA
20 molecule of an isolated mammalian nucleic acid molecule encoding an alternatively spliced prostate-specific membrane antigen. This invention also provides an isolated mammalian cDNA molecule encoding a mammalian alternatively spliced prostate-specific membrane
25 antigen. This invention provides an isolated mammalian RNA molecule encoding a mammalian alternatively spliced prostate-specific membrane antigen.

This invention also encompasses DNAs and cDNAs which
30 encode amino acid sequences which differ from those of PSM' antigen, but which should not produce phenotypic changes. Alternatively, this invention also encompasses DNAs and cDNAs which hybridize to the DNA and cDNA of the subject invention. Hybridization
35 methods are well known to those of skill in the art.

This invention also provides a nucleic acid molecule of

at least 15 nucleotides capable of specifically hybridizing with a sequence of a nucleic acid molecule encoding the prostate-specific membrane antigen. This molecule may either be a DNA or RNA molecule.

5

This invention provides a nucleic acid sequence of at least 15 nucleotides capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located
10 between nucleotide 115 and nucleotide 380.

The nucleic acid molecule capable of specifically
15 hybridizing with a sequence of a nucleic acid molecule encoding the prostate-specific membrane antigen can be used as a probe. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length
20 and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes PSM antigen into suitable vectors, such as plasmids or
25 bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA
30 synthesizers.

RNA probes may be generated by inserting the PSM antigen molecule downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may
35 be produced by incubating the labeled nucleotides with the linearized PSM antigen fragment where it contains an upstream promoter in the presence of the appropriate

RNA polymerase.

For example, high stringent hybridization conditions are selected at about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents, ie. salt or formamide concentration, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one. For Example high stringency may be attained for example by overnight hybridization at about 68°C in a 6x SSC solution, washing at room temperature with 6x SSC solution, followed by washing at about 68°C in a 6x SSC in a 0.6x SSX solution.

Hybridization with moderate stringency may be attained for example by: 1) filter pre-hybridizing and hybridizing with a solution of 3x sodium chloride, sodium citrate (SSC), 50% formamide, 0.1M Tris buffer at Ph 7.5, 5x Denhardt's solution; 2.) pre-hybridization at 37°C for 4 hours; 3) hybridization at 37°C with amount of labelled probe equal to 3,000,000 cpm total for 16 hours; 4) wash in 2x SSC and 0.1% SDS solution; 5) wash 4x for 1 minute each at room temperature at 4x at 60°C for 30 minutes each; and 6) dry and expose to film.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

Moreover, the isolated mammalian nucleic acid molecules encoding a mammalian prostate-specific membrane antigen and the alternatively spliced PSM' are useful for the development of probes to study the tumorigenesis of prostate cancer.

The nucleic acid molecules synthesized above may be used to detect expression of a PSM' antigen by detecting the presence of mRNA coding for the PSM antigen. Total mRNA from the cell may be isolated by many procedures well known to a person of ordinary skill in the art. The hybridizing conditions of the labelled nucleic acid molecules may be determined by routine experimentation well known in the art. The presence of mRNA hybridized to the probe may be determined by gel electrophoresis or other methods known in the art. By measuring the amount of the hybrid made, the expression of the PSM and PSM' antigen by the cell can be determined. The labeling may be radioactive. For an example, one or more radioactive nucleotides can be incorporated in the nucleic acid when it is made.

35

In one embodiment of this invention, nucleic acids are extracted by precipitation from lysed cells and the

mRNA is isolated from the extract using an oligo-dT column which binds the poly-A tails of the mRNA molecules. The mRNA is then exposed to radioactively labelled probe on a nitrocellulose membrane, and the probe hybridizes to and thereby labels complementary mRNA sequences. Binding may be detected by luminescence autoradiography or scintillation counting. However, other methods for performing these steps are well known to those skilled in the art, and the discussion above is merely an example.

The probes are also useful for in-situ hybridization or in order to locate tissues which express this gene, or for other hybridization assays for the presence of this gene or its mRNA in various biological tissues. The in-situ hybridization using a labelled nucleic acid molecule is well known in the art. Essentially, tissue sections are incubated with the labelled nucleic acid molecule to allow the hybridization to occur. The molecule will carry a marker for the detection because it is "labelled", the amount of the hybrid will be determined based on the detection of the amount of the marker and so will the expression of PSM antigen.

This invention further provides isolated PSM' antigen nucleic acid molecule operatively linked to a promoter of RNA transcription. The isolated PSM' antigen sequence can be linked to vector systems. Various vectors including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses are well known to ordinary skilled practitioners. This invention further provides a vector which comprises the isolated nucleic acid molecule encoding for the PSM' antigen.

As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme

to create complementary ends on both molecules which base pair with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other means are also available and known to an ordinary skilled practitioner.

10 Plasmid, p55A-PSM, was deposited on August 14, 1992 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of
15 Microorganism for the Purposes of Patent Procedure. Plasmid, p55A-PSM, was accorded ATCC Accession Number 75294.

20 This invention further provides a host vector system for the production of a polypeptide having the biological activity of the alternatively spliced prostate-specific membrane antigen. These vectors may be transformed into a suitable host cell to form a host cell vector system for the production of a polypeptide
25 having the biological activity of PSM' antigen.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome
30 binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous
35 promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such

vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors
5 are useful to produce cells that express the PSM antigen.

This invention further provides an isolated DNA or cDNA molecule described hereinabove wherein the host cell is
10 selected from the group consisting of bacterial cells (such as E.coli), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

15 This invention provides an isolated polypeptide having the biological activity of an alternatively spliced prostate-specific membrane antigen.

20 This invention further provides a method of producing a polypeptide having the biological activity of the prostate-specific membrane antigen which comprising growing host cells of a vector system containing the PSM' antigen sequence under suitable conditions
25 permitting production of the polypeptide and recovering the polypeptide so produced.

This invention provides a mammalian cell comprising a DNA molecule encoding a mammalian PSM' antigen, such as
30 a mammalian cell comprising a plasmid adapted for expression in a mammalian cell, which comprises a DNA molecule encoding a mammalian PSM' antigen and the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA
35 encoding the mammalian PSM' antigen as to permit expression thereof.

Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Ltk⁺ cells, Cos cells, etc. Expression plasmids such as that described
5 supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, electroporation or DNA encoding the mammalian PSM antigen may be otherwise introduced into mammalian cells, e.g., by microinjection, to obtain
10 mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding a mammalian PSM antigen.

This invention further provides ligands bound to the mammalian PSM' antigen.

15 This invention also provides a therapeutic agent comprising a ligand identified by the above-described method and a cytotoxic agent conjugated thereto. The cytotoxic agent may either be a radioisotope or a
20 toxin. Examples of radioisotopes or toxins are well known to one of ordinary skill in the art.

This invention also provides a method of imaging prostate cancer in human patients which comprises
25 administering to the patients at least one ligand identified by the above-described method, capable of binding to the cell surface of the prostate cancer cell and labelled with an imaging agent under conditions permitting formation of a complex between the ligand
30 and the cell surface PSM' antigen. This invention further provides a composition comprising an effective imaging agent of the PSM' antigen ligand and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to one of ordinary
35 skill in the art. For an example, such a pharmaceutically acceptable carrier can be physiological saline.

Also provided by this invention is a purified mammalian PSM' antigen. As used herein, the term "purified alternatively spliced prostate-specific membrane antigen" shall mean isolated naturally-occurring prostate-specific membrane antigen or protein (purified from nature or manufactured such that the primary, secondary and tertiary conformation, and posttranslational modifications are identical to naturally-occurring material) as well as non-naturally occurring polypeptides having a primary structural conformation (i.e. continuous sequence of amino acid residues). Such polypeptides include derivatives and analogs.

This invention provides an isolated nucleic acid comprising a promoter sequence normally associated with the transcription of a gene encoding a human prostate-specific membrane antigen. In one embodiment regulatory elements are set forth in Figure 15. In another embodiment the promoter is between nucleotide -1 to -641 of Figure 15A.

This invention provides a method to identify such natural ligand or other ligand which can bind to the PSM' antigen. A method to identify the ligand comprises a) coupling the purified mammalian PSM' antigen to a solid matrix, b) incubating the coupled purified mammalian PSM' protein with the potential ligands under the conditions permitting binding of ligands and the purified PSM' antigen; c) washing the ligand and coupled purified mammalian PSM' antigen complex formed in b) to eliminate the nonspecific binding and impurities and finally d) eluting the ligand from the bound purified mammalian PSM' antigen. The techniques of coupling proteins to a solid matrix are well known in the art. Potential ligands may

either be deduced from the structure of mammalian PSM' by other empirical experiments known by ordinary skilled practitioners. The conditions for binding may also easily be determined and protocols for carrying
5 such experimentation are known to those skilled in the art. The ligand-PSM' antigen complex will be washed. Finally, the bound ligand is eluted and characterized. Standard ligands characterization techniques are well known in the art.

10

The above method may also be used to purify ligands from any biological source. For purification of natural ligands in the cell, cell lysates, serum or other biological samples will be used to incubate with
15 the mammalian PSM' antigen bound on a matrix. Specific natural ligand will then be identified and purified as above described.

20

With the protein sequence information, antigenic areas may be identified and antibodies directed against these areas may be generated and targeted to the prostate cancer for imaging the cancer or therapies.

25

This invention provides an antibody directed against the amino acid sequence of a mammalian PSM' antigen.

30

This invention provides a method to select specific regions on the PSM' antigen to generate antibodies. The protein sequence may be determined from the PSM' DNA sequence. Amino acid sequences may be analyzed by
35 methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer of the cell membrane, while hydrophilic regions are located on the

cell surface, in an aqueous environment. Usually, the hydrophilic regions will be more immunogenic than the hydrophobic regions. Therefore the hydrophilic amino acid sequences may be selected and used to generate antibodies specific to mammalian PSM antigen. For an example, hydrophilic sequences of the human PSM antigen shown in hydrophilicity plot may be easily selected. The selected peptides may be prepared using commercially available machines. As an alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen.

Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be produced by in vitro techniques known to a person of ordinary skill in the art. These antibodies are useful to detect the expression of mammalian PSM antigen in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

In one embodiment, peptides Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID No.), Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No.) and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.) of human PSM antigen are selected.

This invention further provides polyclonal and monoclonal antibody(ies) against peptides Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID No.), Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No.) and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.).

This invention provides a method of imaging prostate cancer in human patients which comprises administering to the patient the monoclonal antibody directed against the peptide of the mammalian PSM' antigen capable of
5 binding to the cell surface of the prostate cancer cell and labeled with an imaging agent under conditions permitting formation of a complex between the monoclonal antibody and the cell surface prostate-specific membrane antigen. The imaging agent is a
10 radioisotope such as Indium¹¹¹.

This invention further provides a prostate cancer specific imaging agent comprising the antibody directed against PSM' antigen and a radioisotope conjugated
15 thereto.

This invention also provides a composition comprising an effective imaging amount of the antibody directed against the PSM' antigen and a pharmaceutically
20 acceptable carrier. The methods to determine effective imaging amounts are well known to a skilled practitioner. One method is by titration using different amounts of the antibody.

25 In addition to the standard pharmacophores that can be added to known structures, with the PSM transfectants one can identify potential ligands from combinatorial libraries that might not have been otherwise predicted such combinatorial libraries can be synthetic, peptide,
30 or RNA based.

This invention further provides an immunoassay for measuring the amount of the prostate-specific membrane antigen in a biological sample comprising steps of a)
35 contacting the biological sample with at least one antibody directed against the PSM' antigen to form a complex with said antibody and the prostate-specific

membrane antigen, and b) measuring the amount of the prostate-specific membrane antigen in said biological sample by measuring the amount of said complex. One example of the biological sample is a serum sample.

5

This invention provides a method to purify mammalian prostate-specific membrane antigen comprising steps of a) coupling the antibody directed against the PSM' antigen to a solid matrix; b) incubating the coupled antibody of a) with lysate containing prostate-specific
10 membrane antigen under the condition which the antibody and prostate membrane specific can bind; c) washing the solid matrix to eliminate impurities and d) eluting the prostate-specific membrane antigen from the coupled
15 antibody.

This invention also provides a transgenic nonhuman mammal which comprises the isolated nucleic acid molecule encoding a mammalian PSM' antigen. This
20 invention further provides a transgenic nonhuman mammal whose genome comprises antisense DNA complementary to DNA encoding a mammalian prostate-specific membrane antigen so placed as to be transcribed into antisense mRNA complementary to mRNA encoding the prostate-specific membrane antigen and which hybridizes to mRNA
25 encoding the prostate specific antigen thereby reducing its translation.

Animal model systems which elucidate the physiological
30 and behavioral roles of mammalian PSM' antigen are produced by creating transgenic animals in which the expression of the PSM' antigen is either increased or decreased, or the amino acid sequence of the expressed PSM antigen is altered, by a variety of techniques.
35 Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding a mammalian PSM' antigen, by

is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse
5 stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here
10 only for exemplary purposes.

Another use of the PSM antigen sequence is to isolate homologous gene or genes in different mammals. The gene or genes can be isolated by low stringency screening of
15 either cDNA or genomic libraries of different mammals using probes from PSM sequence. The positive clones identified will be further analyzed by DNA sequencing techniques which are well known to an ordinary person skilled in the art. For example, the detection of
20 members of the protein serine kinase family by homology probing.

This invention provides a method of suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor
25 cells comprising introducing a DNA molecule encoding an alternatively spliced prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell of a subject, in a way that
30 expression of the alternatively spliced prostate specific membrane antigen is under the control of the regulatory element, thereby suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells.
35 The subject may be a mammal or more specifically a human.

In one embodiment, the DNA molecule is operatively linked to a 5' regulatory element forms part of a transfer vector which is inserted into a cell or organism. In addition the vector is capable of replication and expression of the alternatively spliced prostate specific membrane antigen. The DNA molecule can be integrated into a genome of a eukaryotic or prokaryotic cell or in a host cell containing and/or expressing an alternatively spliced prostate specific membrane antigen.

Further, the DNA molecule encoding alternatively spliced prostate specific membrane antigen may be introduced by a bacterial, viral, fungal, animal, or liposomal delivery vehicle. Other means are also available and known to an ordinary skilled practitioner.

Further, the DNA molecule encoding an alternatively spliced prostate specific membrane antigen operatively linked to a promoter or enhancer. A number of viral vectors have been described including those made from various promoters and other regulatory elements derived from virus sources. Promoters consist of short arrays of nucleic acid sequences that interact specifically with cellular proteins involved in transcription. The combination of different recognition sequences and the cellular concentration of the cognate transcription factors determines the efficiency with which a gene is transcribed in a particular cell type.

Examples of suitable promoters include a viral promoter. Viral promoters include: adenovirus promoter, an simian virus 40 (SV40) promoter, a cytomegalovirus (CMV) promoter, a mouse mammary tumor virus (MMTV) promoter, a Malony murine leukemia virus promoter, a murine sarcoma virus promoter, and a Rous

microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these PSM' antigen sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native PSM antigen but does express, for example, an inserted mutant PSM antigen, which has replaced the native PSM antigen in the animal's genome by recombination, resulting in under expression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added PSM antigens, resulting in over expression of the PSM antigens.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as Me medium (16). DNA or cDNA encoding a mammalian PSM antigen is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected

sarcoma virus promoter.

Further, another suitable promoter is a heat shock promoter. Additionally, a suitable promoter is a
5 bacteriophage promoter. Examples of suitable bacteriophage promoters include but not limited to, a T7 promoter, a T3 promoter, an SP6 promoter, a lambda promoter, a baculovirus promoter.

10 Also suitable as a promoter is an animal cell promoter such as an interferon promoter, a metallothionein promoter, an immunoglobulin promoter. A fungal promoter is also a suitable promoter. Examples of fungal promoters include but are not limited to, an
15 ADC1 promoter, an ARG promoter, an ADH promoter, a CYC1 promoter, a CUP promoter, an ENO1 promoter, a GAL promoter, a PHO promoter, a PGK promoter, a GAPDH promoter, a mating type factor promoter. Further, plant cell promoters and insect cell promoters are also
20 suitable for the methods described herein.

This invention provides a method of suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor
25 cells, comprising introducing a DNA molecule encoding an alternatively spliced prostate specific membrane antigen operatively linked to a 5' regulatory element coupled with a therapeutic DNA into a tumor cell of a subject, thereby suppressing or modulating metastatic
30 ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells. The subject may be a mammal or more specifically a human.

Further, the therapeutic DNA which is coupled to the
35 DNA molecule encoding a prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell may code for a cytokine, viral

antigen, or a pro-drug activating enzyme. Other means are also available and known to an ordinary skilled practitioner.

5 In addition, this invention provides a prostate tumor cell, comprising a DNA molecule isolated from mammalian nucleic acid encoding an alternatively spliced mammalian prostate-specific membrane antigen under the control a 5' regulatory element.

10

As used herein, DNA molecules include complementary DNA (cDNA), synthetic DNA, and genomic DNA.

15

This invention provides a therapeutic vaccine for preventing human prostate tumor growth or stimulation of prostate tumor cells in a subject, comprising administering an effective amount to the prostate cell, and a pharmaceutical acceptable carrier, thereby preventing the tumor growth or stimulation of tumor cells in the subject. Other means are also available and known to an ordinary skilled practitioner.

20

25

This invention provides a method of detecting hematogenous micrometastatic tumor cells of a subject, comprising (A) performing nested polymerase chain reaction (PCR) on blood, bone marrow or lymph node samples of the subject using the prostate specific membrane antigen primers or alternatively spliced prostate specific antigen primers, and (B) verifying micrometastases by DNA sequencing and Southern analysis, thereby detecting hematogenous micrometastatic tumor cells of the subject. The subject may be a mammal or more specifically a human.

30

35

The micrometastatic tumor cell may be a prostatic cancer and the DNA primers may be derived from prostate specific antigen. Further, the subject may be

administered with simultaneously an effective amount of hormones, so as to increase expression of prostate specific membrane antigen. Further, growth factors or cytokine may be administered in separately or in
5 conjunction with hormones. Cytokines include, but are not limited to: transforming growth factor beta, epidermal growth factor (EGF) family, fibroblast growth factors, hepatocyte growth factor, insulin-like growth factors, B-nerve growth factor, platelet-derived growth
10 factor, vascular endothelial growth factor, interleukin 1, IL-1 receptor antagonist, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, IL-6 soluble receptor, interleukin 7, interleukin 8, interleukin 9, interleukin 10, interleukin 11,
15 interleukin 12, interleukin 13, angiogenin, chemokines, colony stimulating factors, granulocyte-macrophage colony stimulating factors, erythropoietin, interferon, interferon gamma, leukemia inhibitory factor, oncostatin M, pleiotrophin, secretory leukocyte
20 protease inhibitor, stem cell factor, tumor necrosis factors, adhesion molecule, and soluble tumor necrosis factor (TNF) receptors.

This invention provides a method of abrogating the
25 mitogenic response due to transferrin, comprising introducing a DNA molecule encoding prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell, the expression of which gene is directly associated with a defined pathological
30 effect within a multicellular organism, thereby abrogating mitogen response due to transferrin. The tumor cell may be a prostate cell.

This invention provides a method of determining
35 prostate cancer progression in a subject which comprises: a) obtaining a suitable prostate tissue sample; b) extracting RNA from the prostate tissue

sample; c) performing a RNase protection assay on the RNA thereby forming a duplex RNA-RNA hybrid; d) detecting PSM and PSM' amounts in the tissue sample; e) calculating a PSM/PSM' tumor index, thereby determining prostate cancer progression in the subject. In-situ hybridization may be performed in conjunction with the above detection method.

This invention provides a method of detecting prostate cancer in a subject which comprises: (a) obtaining from a subject a prostate tissue sample; (b) treating the tissue sample so as to separately recover nucleic acid molecules present in the prostate tissue sample; (c) contacting the resulting nucleic acid molecules with multiple pairs of single-stranded labeled oligonucleotide primers, each such pair being capable of specifically hybridizing to the tissue sample, under hybridizing conditions; (d) amplifying any nucleic acid molecules to which a pair of primers hybridizes so as to obtain a double-stranded amplification product; (e) treating any such double-stranded amplification product so as to obtain single-stranded nucleic acid molecules therefrom; (f) contacting any resulting single-stranded nucleic acid molecules with multiple single-stranded labeled oligonucleotide probes, each such probe containing the same label and being capable of specifically hybridizing with such tissue sample, under hybridizing conditions; (g) contacting any resulting hybrids with an antibody to which a marker is attached and which is capable of specifically forming a complex with the labeled-probe, when the probe is present in such a complex, under complexing conditions; and (h) detecting the presence of any resulting complexes, the presence thereof being indicative of prostate cancer in a subject.

This invention provides a method of enhancing antibody

based targeting of PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient b-FGF in sufficient amount to cause upregulation of PSM' expression.

5

This invention provides a method of enhancing antibody based targeting of PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient TGF in sufficient amount to cause upregulation of PSM expression or PSM'.

10

This invention provides a method of enhancing antibody based targeting of PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient EGF in sufficient amount to cause upregulation of PSM' expression.

15

This invention provides a method of detecting in a sample the presence of a nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen which comprises: a) obtaining a suitable sample; b) extracting RNA from the sample; c) contacting the RNA with reverse transcriptase under suitable conditions to obtain a cDNA; d) contacting the cDNA under hybridizing conditions with two oligonucleotide primers, i) the first primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 3' of nucleotide 114 of such DNA sequence, with the proviso that the 3' end of the primer does not hybridize to any sequence located 5' of nucleotide 114, and ii) the second primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 5' of nucleotide 381 of such DNA sequence, with the proviso that the 5' end of the primer does not hybridize to any

20

25

30

35

sequence located 3' of nucleotide 381; d) amplifying any cDNA to which the primers hybridize to so as to obtain amplification product; e) determining the size of the amplification product; f) comparing the size of the amplification product to the size of the amplification product known to be obtained using the same primers with a non alternatively spliced human prostate specific membrane antigen, wherein a smaller amplification product is indicative of the presence of the alternatively spliced prostate specific membrane antigen, so as to thereby detect the presence of the alternatively spliced human prostate-specific membrane antigen in the sample.

In one embodiment the suitable sample may be any bodily tissue or fluid which includes but is not limited to: blood, bone marrow, and lymph nodes.

In one embodiment the primers are at least 14-25 nucleotides in length. In another embodiment the primers are at least 15 nucleotides in length. In another embodiment the primers are 15 nucleotides in length. In another embodiment multiple primers are used. Construction of primers which hybridize and hybridizing conditions are known to those skilled in the art. For example, based on Figure 18 one skilled in the art may construct primers which hybridize to the prostate specific membrane antigen before nucleotide 114 and after nucleotide 381.

Further, a method of determining the amount of the amplification product or products (i.e. 2 or more bands) as well as the ratio of each product is known to those skilled in the art. For example, the amount of prostate specific membrane antigen or alternatively spliced prostate specific membrane antigen may be determined by density, binding radiolabeled probes,

autoradiography, UV spectrography, spectrophotometer, optical scan , and phospho-imaging.

5 This invention provides a method of detecting a prostate tumor cell in a subject which comprises:
which comprises: a) obtaining a suitable sample; b) extracting RNA from the sample; c) contacting the RNA with reverse transcriptase under suitable conditions to obtain a cDNA; d) contacting the cDNA under hybridizing
10 conditions with two oligonucleotide primers, i) the first primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 3' of nucleotide 114 of such DNA sequence, with the proviso
15 that the 3' end of the primer does not hybridize to any sequence located 5' of nucleotide 114, and ii) the second primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 5' of
20 nucleotide 381 of such DNA sequence, with the proviso that the 5' end of the primer does not hybridize to any sequence located 3' of nucleotide 381; d) amplifying any cDNA to which the primers hybridize to so as to obtain amplification product; e) determining the amount
25 of the amplification product; f) comparing the amount of the amplification product to the amount of the amplification product known to be obtained using the same primers with a non alternatively spliced human prostate specific membrane antigen, wherein a greater
30 amount of the prostate specific membrane antigen is indicative of a prostate tumor cell in the subject, so as to thereby detect prostate tumor cell in the subject.

35 In PCR techniques, oligonucleotide primers complementary to the two 3' borders of the DNA of the prostate specific membrane (PSM) antigen to be

amplified are synthesized. The polymerase chain reaction is then carried out using the two primers. See *PCR Protocols: A Guide to Methods and Applications*. Hybridization of PSM antigen DNA to the above nucleic acid probes can be performed by a Southern blot under stringent hybridization conditions as described herein.

Oligonucleotides for use as probes or PCR primers are chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage and Carruthers using an automated synthesizer, as described in Needham-VanDevanter. Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson, J.D. and Regnier, F.E. The sequence of the synthetic oligonucleotide can be verified using the chemical degradation method of Maxam, A.M. and Gilbert, W.

Accepted means for conducting hybridization assays are known and general overviews of the technology can be had from a review of: *Nucleic Acid Hybridization: A Practical Approach*; *Hybridization of Nucleic Acids Immobilized on Solid Supports*; *Analytical Biochemistry* and Innis et al., *PCR Protocols*.

If PCR is used in conjunction with nucleic acid hybridization, primers are designed to target a specific portion of the nucleic acid of DNA of the PSM antigen. From the information provided herein, those of skill in the art will be able to select appropriate specific primers.

It will be apparent to those of ordinary skill in the art that a convenient method for determining whether a probe is specific for PSM antigen or PSM' antigen utilizes a Southern blot (or Dot blot). Briefly, to

identify a target specific probe DNA is isolated from the PSM or PSM' antigen. Test DNA is transferred to a solid (e.g., charged nylon) matrix. The probes are labelled following conventional methods. Following denaturation and/or prehybridization steps known in the art, the probe is hybridized to the immobilized DNAs under stringent conditions. Stringent hybridization conditions will depend on the probe used and can be estimated from the calculated T_m (melting temperature) of the hybridized probe (see, e.g., Sambrook for a description of calculation of the T_m). For radioactively-labeled DNA or RNA probes an example of stringent hybridization conditions is hybridization in a solution containing denatured probe and 5x SSC at 65°C for 8-24 hours followed by washes in 0.1x SSC, 0.1% SDS (sodium dodecyl sulfate) at 50-65°C. In general, the temperature and salt concentration are chosen so that the post hybridization wash occurs at a temperature that is about 5°C below the T_m of the hybrid. Thus for a particular salt concentration the temperature may be selected that is 5°C below the T_m or conversely, for a particular temperature, the salt concentration is chosen to provide a T_m for the hybrid that is 5°C warmer than the wash temperature. Following stringent hybridization and washing, a probe that hybridizes to the PSM antigen or PSM' antigen as evidenced by the presence of a signal associated with the appropriate target and the absence of a signal from the non-target nucleic acids, is identified as specific. It is further appreciated that in determining probe specificity and in utilizing the method of this invention a certain amount of background signal is typical and can easily be distinguished by one of skill from a specific signal. Two fold signal over background is acceptable.

This invention provides a therapeutic agent comprising

antibodies or ligand(s) directed against PSM' antigen and a cytotoxic agent conjugated thereto or antibodies linked enzymes which activate prodrug to kill the tumor. The cytotoxic agent may either be a
5 radioisotope or toxin.

This invention provides a compound comprising a conjugate of a cytotoxic agent and one or more amino acid residues, wherein each amino acid residue is
10 glutamate or aspartate. In one embodiment the amino acid residues alternate.

Examples of cytotoxic chemotherapeutic agents or antineoplastic agents) include, but are not limited to
15 the following: Antimetabolites: Denopterin, Edatrexate, Piritrexim, Pteropterin, Tomudex, Tremetrexate, Cladribine, Fludarabine, 6-Mercaptopurine, Thiamiprine, Thioguanine, Ancitabine, Azacitidine, 6-Azaauridine, Carmofur, Cytarabine,
20 Doxifluride, Emitefur, Enocitabine, Floxuridine, Fluorocit, Gemcitabine, and Tegafur.

Alkaloids: Docetaxel, Etoposide, Irinotecan, Paclitaxel, Teniposide, Topotecan, Vinblastine,
25 Vincristine, and Vindesine.

Alkylating agents: Alkyl Sulfonates: Busulfan, Improsulfan, Piposulfan, Aziridines, Benzodepa, Carboquone, Meuredopa, Uredopa, Ethylenimines and
30 Methylmelamines, Altretamine, Triethylenemelamine, Triethylenephosphoramidate, Triethylenethiophosphoramidate, Chlorambucil, Chlornaphazine, Cyclophosphamide, Estramustine, Ifosfamide, Mechlorethamine, Mechlorethamine Oxide Hydrochloride, Melphalan,
35 Novembiechin, Perfosfamide, Phenesterine, Prednimustine, Trofosfamide, Uracil Mustard, Carmustine, Chlorozotocin, Fotemustine, Lomustine,

Nimustine, Ranimustine, Dacarbazine, Mannomustine,
Mitbronitol, Mitolactol, Pipobroman, Temozolomide,
Antibiotics and Analogs: Aclacinomycins, Actinomycin,
Anthramycin, Azaserine, Bleomycins, Cactinomycin,
5 Carubicin, Carzinophilin, Chromomycins, Dactinomycin,
Caunorubicin, 6-Diazo-5-oxo-L-norleucine, Doxorubicin,
Epirubicin, Idarubicin, Menogaril, Mitomycins,
Mycophenolic Acid, Nogalamycin, Olivomycins,
Peplomycin, Pirarubicin, Plicamycin, Porfiromycin,
10 Puromycin, Streptonigrin, Streptozocin, Tubercidin,
Zinostatin, Zorubicin, and L-Asparaginase.

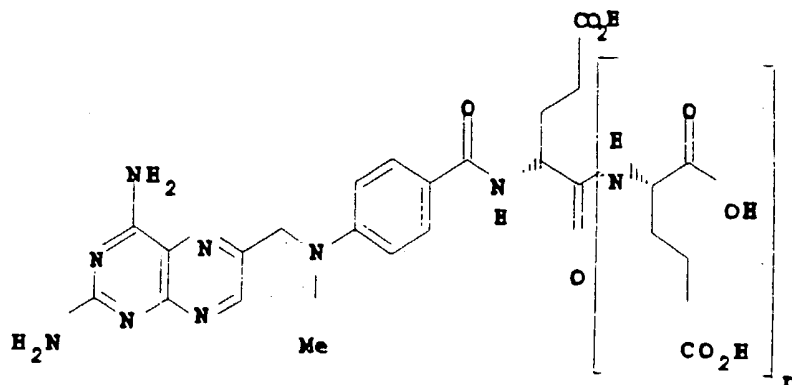
Immunomodulators: Interferon, Interferon-B, Interferon-Y,
Interleukin-2, Lentinan, Propagermanium, PSK,
15 Roquinimex, Sizofran, and Ubenimex. Platinum complexes:
Carboplatin, Cisplatin, Miboplatin, and Oxaliplatin.

Others: Aceglatone, Amsacrine, Bisantrene,
Defoosfamide, Demecolcine, Diaziquone, Eflornithine,
20 Eliptinium Acetate, Etoglucid, Fenertinide, Gallium
Nitrate, Hydroxyurea, Lonidamine, Miltefosine,
Mitoguazone, Mitoxantrone, Mopidamol, Nitracirine,
Pentostatin, Phenamet, Podophyllinic Acid 2-Ethyl-
hydrazide, Procarbazine, Razoxane, Sobuzoxane,
25 Spirogermanium, Tenuazonic Acid, Triaziquone, Urethan,
Calusterone, Dromostanolone, Epitiostanol,
Mepitiostane, Testolactone, Amiglutehimide, Mitotane,
Trilostane, Droloxifene, Tamoxifen, Toremifene,
Aminogluthethimide, Anastrozole, Fadrozole, Formestane,
30 Letrozole, Fosfestrol, Hexestrol, Polyestradiol
Phosphate, Buserlin, Goserlin, Leuprolide, Triptorelin,
Chlormadinone Acetate, Medroxyprogesterone, Megerstrol
Acetate, Melengestrol, Porfimer Sodium, Americium,
Chromic Phosphate, Radioactive Cobalt, I-Ehtiodized
35 Oil, Gold, Radioactive, Colloidal, Iobenguane, Radium,
Radon, Sodium Iodide, Sodium Phosphate, Radioactive,
Batimastat, Folinic Acid, Amifostine, Etanidazole,

Etamidozole, and Mesna.

This invention provides a compound, wherein the compound has the structure:

5



wherein n is an integer from 1-10 inclusive.

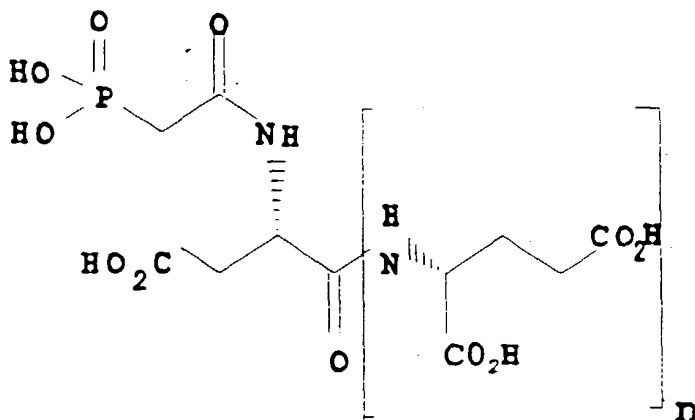
10

In one embodiment glutamate may be in L or D to form either 4-amino-N¹⁰-methyl pteroyl-L-glutamate or 4-amino-N¹⁰-methyl pteroyl-D-glutamate. In another embodiment aspartate may substitute the glutamate to form 4-amino-N¹⁰-methyl pteroyl-L-aspartate. In another embodiment aspartate may substitute the glutamate to form 4-amino-N¹⁰-methyl pteroyl-D-aspartate. In another embodiment the 4-amino-N¹⁰-methyl pteroyl may have alternating glutamate or aspartat moieties. The glutamate or aspartate are bound to the methotrexate at the alpha carbon position of methotrexate.

20

25

This invention provides a compound, wherein the compound has the structure:



5

wherein n is an integer from 1-10 inclusive.

In one embodiment glutamate may be in the L or D to
 10 form either N-phosphonoacetyl-L-aspartyl (PALA)-
 glutamate or N-phosphonoacetyl-D-aspartyl-glutamate. In
 another embodiment aspartate may substitute the
 glutamate to form N-phosphonoacetyl-L-aspartyl-
 aspartate. In another embodiment the 4-amino-N¹⁰-methyl
 15 pteroyl may have alternating glutamate or aspartate
 moieties.

20

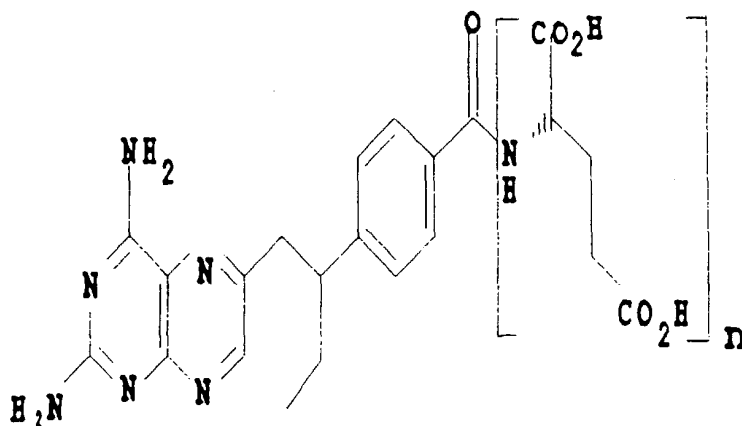
25

This invention provides a compound, wherein the compound has the structure:

5

10

15



wherein n is an integer from 1-10 inclusive.

20 In one embodiment glutamate may be in the L or D to form either 4-amino-10-ethyl-10-deazapteroyl (EDAM) - L-glutamate or 4-amino-10-ethyl-10-deazapteroyl-D-glutamate. In another embodiment aspartate may substitute the glutamate to form 4-amino-10-ethyl-10-deazapteroyl-L-aspartate. In another embodiment the 4-amino-10-ethyl-10-deazapteroyl may have alternating
25 glutamate or aspartat moieties.

This invention provides a pharmaceutical composition comprising any of the above compounds in a
30 therapeutically effective amount and a pharmaceutically acceptable carrier.

This invention provides a method of making prostate cells susceptible to a cytotoxic agent, which comprises
35 contacting the prostate cells with any of the above compounds in an amount effective to render the prostate cells susceptible to the cytotoxic chemotherapeutic

agent.

This invention provides a pharmaceutical composition comprising an effective amount the alternatively spliced PSM' and a carrier or diluent. Further, this invention provides a method for administering to a subject, preferably a human, the pharmaceutical composition. Further, this invention provides a composition comprising an amount of the alternatively spliced PSM' and a carrier or diluent. Specifically, this invention may be used as a food additive.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each subject.

In one embodiment the therapeutic effective amount is 100-10,000 mg/m² IV with rescue. In another embodiment the therapeutic effective amount is 300-1000 mg/m² IV or continuous infusion. In another embodiment the therapeutic effective amount is 100 mg/m² IV continuous infusion. In another embodiment the therapeutic effective amount is 40-75 mg/m² rapidly. In another embodiment the therapeutic effective amount is 30 mg/m² for 3 days by continuous IV.

Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration.

35

As used herein administration means a method of administering to a subject. Such methods are well

known to those skilled in the art and include, but are not limited to, administration topically, parenterally, orally, intravenously, intramuscularly, subcutaneously or by aerosol. Administration of PSM may be effected
5 continuously or intermittently.

The pharmaceutical formulations or compositions of this invention may be in the dosage form of solid, semi-solid, or liquid such as, e.g., suspensions, aerosols
10 or the like. Preferably the compositions are administered in unit dosage forms suitable for single administration of precise dosage amounts. The compositions may also include, depending on the formulation desired, pharmaceutically-acceptable, non-
15 toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents
20 are distilled water, physiological saline, Ringer's solution, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants; or nontoxic, nontherapeutic, nonimmunogenic stabilizers
25 and the like. Effective amounts of such diluent or carrier are those amounts which are effective to obtain a pharmaceutically acceptable formulation in terms of solubility of components, or biological activity, etc

30

This invention also provides a method of detecting a subject with cancer comprising a) contacting a cell of the neo-vasculature of a subject with a ligand which
35 binds to the extracellular domain of the PSM antigen under conditions permitting formation of a complex; and b) detecting the complex with a labelled imaging

agent, thereby detecting a subject with cancer.

5 In one embodiment the cancer is, but is not limited to:
kidney, colon, or bladder. In one embodiment the
ligand is CYT-356. In another embodiment the ligand is
any antibody, monoclonal or polyclonal which binds to
the extracellular domain of PSM antigen. In one
embodiment the cells of endothelial cells of the neo-
vasculature of a subject with cancer.

10

This invention will be better understood from the
Experimental Details which follow. However, one
skilled in the art will readily appreciate that the
specific methods and results discussed are merely
15 illustrative of the invention as described more fully
in the claims which follow thereafter.

EXPERIMENTAL DETAILS

EXAMPLE 1:

5 EXPRESSION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN

A 2.65 kb complementary DNA encoding PSM was cloned. Immunohistochemical analysis of the LNCaP, DU-145, and PC-3 prostate cancer cell lines for PSM expression using the 7E11-C5.3 antibody reveals intense staining in the LNCaP cells, with no detectable expression in both the DU-145 and PC-3 cells. Coupled in-vitro transcription/ translation of the 2.65 kb full-length PSM cDNA yields an 84 kDa protein corresponding to the predicted polypeptide molecular weight of PSM. Post-translational modification of this protein with pancreatic canine microsomes yields the expected 100 kDa PSM antigen. Following transfection of PC-3 cells with the full-length PSM cDNA in a eukaryotic expression vector applicant's detect expression of the PSM glycoprotein by Western analysis using the 7E11-C5.3 monoclonal antibody. Ribonuclease protection analysis demonstrates that the expression of PSM mRNA is almost entirely prostate-specific in human tissues. PSM expression appears to be highest in hormone-deprived states and is hormonally modulated by steroids, with DHT down regulating PSM expression in the human prostate cancer cell line LNCaP by 8-10 fold, testosterone down regulating PSM by 3-4 fold, and corticosteroids showing no significant effect. Normal and malignant prostatic tissues consistently show high PSM expression, whereas heterogeneous, and at times absent, from expression of PSM in benign prostatic hyperplasia. LNCaP tumors implanted and grown both orthotopically and subcutaneously in nude mice, abundantly express PSM providing an excellent in-vivo model system to study the regulation and modulation of

PSM expression.

Materials and Methods:

- 5 **Cells and Reagents:** The LNCaP, DU-145, and PC-3 cell
lines were obtained from the American Type Culture
Collection. Details regarding the establishment and
characteristics of these cell lines have been
previously published. Unless specified otherwise,
10 LNCaP cells were grown in RPMI 1640 media supplemented
with L-glutamine, nonessential amino acids, and 5%
fetal calf serum (Gibco-BRL, Gaithersburg, MD.) in a
CO₂ incubator at 37C. DU-145 and PC-3 cells were grown
in minimal essential medium supplemented with 10% fetal
15 calf serum. All cell media were obtained from the
MSKCC Media Preparation Facility. Restriction and
modifying enzymes were purchased from Gibco-BRL unless
otherwise specified.
- 20 **Immunohistochemical Detection of PSM:** Avidin-biotin
method of detection was employed to analyze prostate
cancer cell lines for PSM antigen expression. Cell
cytospins were made on glass slides using 5x10⁴
cells/100ul per slide. Slides were washed twice with
25 PBS and then incubated with the appropriate suppressor
serum for 20 minutes. The suppressor serum was drained
off and the cells were incubated with diluted 7E11-C5.3
(5g/ml) monoclonal antibody for 1 hour. Samples were
then washed with PBS and sequentially incubated with
30 secondary antibodies for 30 minutes and with avidin-
biotin complexes for 30 minutes. Diaminobenzidine
served as the chromogen and color development followed
by hematoxylin counterstaining and mounting. Duplicate
cell cytospins were used as controls for each
35 experiment. As a positive control, the anti-
cytokeratin monoclonal antibody CAM 5.2 was used
following the same procedure described above. Human EJ

bladder carcinoma cells served as a negative control.

In-Vitro Transcription/Translation of PSM Antigen:

Plasmid 55A containing the full length 2.65 kb PSM cDNA
5 in the plasmid pSPORT 1 (Gibco-BRL) was transcribed in-
vitro using the Promega TNT system (Promega Corp.
Madison, WI). T7 RNA polymerase was added to the cDNA
in a reaction mixture containing rabbit reticulocyte
lysate, an amino acid mixture lacking methionine,
10 buffer, and ³⁵S-Methionine (Amersham) and incubated at
30C for 90 minutes. Post-translational modification of
the resulting protein was accomplished by the addition
of pancreatic canine microsomes into the reaction
mixture (Promega Corp. Madison, WI.). Protein products
15 were analyzed by electrophoresis on 10% SDS-PAGE gels
which were subsequently treated with Amplify
autoradiography enhancer (Amersham, Arlington Heights,
IL.) according to the manufacturers instructions and
dried at 80C in a vacuum dryer. Gels were
20 autoradiographed overnight at -70C using Hyperfilm MP
(Amersham).

Transfection of PSM into PC-3 Cells: The full length
PSM cDNA was subcloned into the pREP7 eukaryotic
25 expression vector (Invitrogen, San Diego, CA.).
Plasmid DNA was purified from transformed DH5-alpha
bacteria (Gibco-BRL) using Qiagen maxi-prep plasmid
isolation columns (Qiagen Inc., Chatsworth, CA.).
Purified plasmid DNA (6-10g) was diluted with 900ul of
30 Optimem media (Gibco-BRL) and mixed with 30ul of
Lipofectin reagent (Gibco-BRL) which had been
previously diluted with 900l of Optimem media. This
mixture was added to T-75 flasks of 40-50% confluent
PC-3 cells in Optimem media. After 24-36 hours, cells
35 were trypsinized and split into 100mm dishes
containing RPMI 1640 media supplemented with 10% fetal
calf serum and 1 mg/ml of Hygromycin B (Calbiochem, La

Jolla, CA.). The dose of Hygromycin B used was previously determined by a time course/dose response cytotoxicity assay. Cells were maintained in this media for 2-3 weeks with changes of media and Hygromycin B every 4-5 days until discrete colonies appeared. Colonies were isolated using 6mm cloning cylinders and expanded in the same media. As a control, PC-3 cells were also transfected with the pREP7 plasmid alone. RNA was isolated from the transfected cells and PSM mRNA expression was detected by both RNase Protection analysis (described later) and by Northern analysis.

Western Blot Detection of PSM Expression: Crude protein lysates were isolated from LNCaP, PC-3, and PSM-transfected PC-3 cells as previously described. LNCaP cell membranes were also isolated according to published methods. Protein concentrations were quantitated by the Bradford method using the BioRad protein reagent kit (BioRad, Richmond, CA.). Following denaturation, 20 μ g of protein was electrophoresed on a 10% SDS-PAGE gel at 25 mA for 4 hours. Gels were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA.) overnight at 4C. Membranes were blocked in 0.15M NaCl/0.01M Tris-HCl (TS) plus 5% BSA followed by a 1 hour incubation with 7E11-C5.3 monoclonal antibody (10 μ g/ml). Blots were washed 4 times with 0.15M NaCl/0.01M Tris-HCl/0.05% Triton-X 100 (TS-X) and incubated for 1 hour with rabbit anti-mouse IgG (Accurate Scientific, Westbury, N.Y.) at a concentration of 10 μ g/ml.

Blots were then washed 4 times with TS-X and labeled with ¹²⁵I-Protein A (Amersham, Arlington Heights, IL.) at a concentration of 1 million cpm/ml. Blots were then washed 4 times with TS-X and dried on Whatman 3MM paper, followed by overnight autoradiography at -70C

using Hyperfilm MP (Amersham).

Orthotopic and Subcutaneous LNCaP Tumor Growth in Nude Mice: LNCaP cells were harvested from sub-confluent cultures by a one minute exposure to a solution of 0.25% trypsin and 0.02% EDTA. Cells were resuspended in RPMI 1640 media with 5% fetal bovine serum, washed and diluted in either Matrigel (Collaborative Biomedical Products, Bedford, MA.) or calcium and magnesium-free Hank's balanced salt solution (HBSS). Only single cell suspensions with greater than 90% viability by trypan blue exclusion were used for in vivo injection. Male athymic Swiss (nu/nu) nude mice 4-6 weeks of age were obtained from the Memorial Sloan-Kettering Cancer Center Animal Facility. For subcutaneous tumor cell injection one million LNCaP cells resuspended in 0.2 mls. of Matrigel were injected into the hindlimb of each mouse using a disposable syringe fitted with a 28 gauge needle. For orthotopic injection, mice were first anesthetized with an intraperitoneal injection of Pentobarbital and placed in the supine position. The abdomen was cleansed with Betadine and the prostate was exposed through a midline incision. 2.5 million LNCaP tumor cells in 0.1 ml. were injected directly into either posterior lobe using a 1 ml disposable syringe and a 28 gauge needle. LNCaP cells with and without Matrigel were injected. Abdominal closure was achieved in one layer using Autoclip wound clips (Clay Adams, Parsippany, N.J.). Tumors were harvested in 6-8 weeks, confirmed histologically by faculty of the Memorial Sloan-Kettering Cancer Center Pathology Department, and frozen in liquid nitrogen for subsequent RNA isolation.

RNA Isolation: Total cellular RNA was isolated from cells and tissues by standard techniques (3 and 17) as well as by using RNazol B (Cinna/Biotechx, Houston,

TX.). RNA concentrations and quality were assessed by
UV spectroscopy on a Beckman DU 640 spectrophotometer
and by gel analysis. Human tissue total RNA samples
were purchased from Clontech Laboratories, Inc., Palo
5 Alto, CA.

Ribonuclease Protection Assays: A portion of the PSM
cDNA was subcloned into the plasmid vector pSPORT 1
(Gibco-BRL) and the orientation of the cDNA insert
10 relative to the flanking T7 and SP6 RNA polymerase
promoters was verified by restriction analysis. Linearization of this plasmid upstream of the PSM
insert followed by transcription with SP6 RNA
polymerase yields a 400 nucleotide antisense RNA probe,
15 of which 350 nucleotides should be protected from RNase
digestion by PSM RNA. This probe was used in Figure
20. Plasmid IN-20, containing a 1 kb partial PSM cDNA
in the plasmid pCR II (Invitrogen) was also used for
riboprobe synthesis. IN-20 linearized with Xmn I
20 (Gibco-BRL) yields a 298 nucleotide anti-sense RNA
probe when transcribed using SP6 RNA polymerase, of
which 260 nucleotides should be protected from RNase
digestion by PSM mRNA. This probe was used in Figures
21 and 22. Probes were synthesized using SP6 RNA
25 polymerase (Gibco-BRL), rNTPs (Gibco-BRL), RNasin
(Promega), and ³²P-rCTP (NEN, Wilmington, DE.) according
to published protocols (44). Probes were purified over
NENSORB 20 purification columns (NEN) and approximately
1 million cpm of purified, radiolabeled PSM probe was
30 mixed with 10 μ of each RNA and hybridized overnight at
45C using buffers and reagents from the RPA II kit
(Ambion, Austin, TX). Samples were processed as per
manufacturer's instructions and analyzed on 5%
polyacrilamide/7M urea denaturing gels using Seq ACRYL
35 reagents (ISS, Natick, MA.). Gels were pre-heated to
55C and run for approximately 1-2 hours at 25 watts.
Gels were then fixed for 30 minutes in 10% methanol/10%

acetic acid, dried onto Whatman 3MM paper at 80C in a BioRad vacuum dryer and autoradiographed overnight with Hyperfilm MP (Amersham). Quantitation of PSM expression was determined by using a scanning laser
5 densitometer (LKB, Piscataway, NJ.).

Steroid Modulation Experiment: LNCaP cells (2 million) were plated onto T-75 flasks in RPMI 1640 media supplemented with 5% fetal calf serum and grown 24
10 hours until approximately 30-40% confluent. Flasks were then washed several times with phosphate-buffered saline and RPMI medium supplemented with 5% charcoal-extracted serum was added. Cells were then grown for another 24 hours, at which time dihydrotestosterone,
15 testosterone, estradiol, progesterone, and dexamethasone (Steraloids Inc., Wilton, NH.) were added at a final concentration of 2 nM. Cells were grown for another 24 hours and RNA was then harvested as previously described and PSM expression analyzed by
20 ribonuclease protection analysis.

Experimental Results

25 Immunohistochemical Detection of PSM: Using the 7E11-C5.3 anti-PSM monoclonal antibody, PSM expression is clearly detectable in the LNCaP prostate cancer cell line, but not in the PC-3 and DU-145 cell lines (Figures 17A-17C). All normal and malignant prostatic
30 tissues analyzed stained positively for PSM expression.

In-Vitro Transcription/Translation of PSM Antigen: As shown in Figure 18, coupled in-vitro transcription/translation of the 2.65 kb full-length PSM cDNA yields
35 an 84 kDa protein species in agreement with the expected protein product from the 750 amino acid PSM open reading frame. Following post-translational

modification using pancreatic canine microsomes were obtained a 100 kDa glycosylated protein species consistent with the mature, native PSM antigen.

- 5 **Detection of PSM Antigen in LNCaP Cell Membranes and Transfected PC-3 Cells:** PC-3 cells transfected with the full length PSM cDNA in the pREP7 expression vector were assayed for expression of SM mRNA by Northern analysis. A clone with high PSM mRNA expression was selected for PSM antigen analysis by Western blotting using the 7E11-C5.3 antibody. In Figure 19, the 100 kDa PSM antigen is well expressed in LNCaP cell lysate and membrane fractions, as well as in PSM-transfected PC-3 cells but not in native PC-3 cells. This detectable expression in the transfected PC-3 cells proves that the previously cloned 2.65 kb PSM cDNA encodes the antigen recognized by the 7E11-C5.3 anti-prostate monoclonal antibody.
- 10
- 15
- 20 **PSM mRNA Expression:** Expression of PSM mRNA in normal human tissues was analyzed using ribonuclease protection assays. Tissue expression of PSM appears predominantly within the prostate, with very low levels of expression detectable in human brain and salivary gland (Figure 20). No detectable PSM mRNA expression was evident in non-prostatic human tissues when analyzed by Northern analysis. On occasion it is noted that detectable PSM expression in normal human small intestine tissue, however this mRNA expression is variable depending upon the specific riboprobe used. All samples of normal human prostate and human prostatic adenocarcinoma assayed have revealed clearly detectable PSM expression, whereas generally decreased or absent expression of PSM in tissues exhibiting benign hyperplasia (Figure 21). In human LNCaP tumors grown both orthotopically and subcutaneously in nude mice abundant PSM expression with or without the use of
- 25
- 30
- 35

matrigel, which is required for the growth of subcutaneously implanted LNCaP cells was detected (Figure 21). PSM mRNA expression is distinctly modulated by the presence of steroids in physiologic doses (Figure 22). DHT downregulated expression by 8-10 fold after 24 hours and testosterone diminished PSM expression by 3-4 fold. Estradiol and progesterone also downregulated PSM expression in LNCaP cells, perhaps as a result of binding to the mutated androgen receptor known to exist in the LNCaP cell. Overall, PSM expression is highest in the untreated LNCaP cells grown in steroid-depleted media, a situation that simulates the hormone-deprived (castrate) state in-vivo. This experiment was repeated at steroid dosages ranging from 2-200 nM and at time points from 6 hours to 7 days with similar results; maximal downregulation of PSM mRNA was seen with DHT at 24 hours at doses of 2-20 nM.

20 Experimental Discussion

Previous research has provided two valuable prostatic bio-markers, PAP and PSA, both of which have had a significant impact on the diagnosis, treatment, and management of prostate malignancies. The present work describing the preliminary characterization of the prostate-specific membrane antigen (PSM) reveals it to be a gene with many interesting features. PSM is almost entirely prostate-specific as are PAP and PSA, and as such may enable further delineation of the unique functions and behavior of the prostate. The predicted sequence of the PSM protein (30) and its presence in the LNCaP cell membrane as determined by Western blotting and immunohistochemistry, indicate that it is an integral membrane protein. Thus, PSM provides an attractive cell surface epitope for antibody-directed diagnostic imaging and cytotoxic

targeting modalities . The ability to synthesize the PSM antigen in-vitro and to produce tumor xenografts maintaining high levels of PSM expression provides us with a convenient and attractive model system to further study and characterize the regulation and modulation of PSM expression. Also, the high level of PSM expression in the LNCaP cells provides an excellent in-vitro model system. Since PSM expression is hormonally-responsive to steroids and may be highly expressed in hormone-refractory disease. The detection of PSM mRNA expression in minute quantities in brain, salivary gland, and small intestine warrants further investigation, although these tissues were negative for expression of PSM antigen by immunohistochemistry using the 7E11-C5.3 antibody. In all of these tissues, particularly small intestine, mRNA expression using a probe corresponding to a region of the PSM cDNA near the 3' end, whereas expression when using a 5' end PSM probe was not detected. These results may indicate that the PSM mRNA transcript undergoes alternative splicing in different tissues.

Applicants approach is based on prostate tissue specific promotor: enzyme or cytokine chimeras. Promotor specific activation of prodrugs such as non toxic gancyclovir which is converted to a toxic metabolite by herpes simplex thymidine kinase or the prodrug 4-(bis(2chloroethyl)amino)benzoyl-L-glutamic acid to the benzoic acid mustard alkylating agent by the pseudomonas carboxy peptidase G2 was examined. As these drugs are activated by the enzyme (chimera) specifically in the tumor the active drug is released only locally in the tumor environment, destroying the surrounding tumor cells. Promotor specific activation of cytokines such as IL-12, IL-2 or GM-CSF for activation and specific antitumor vaccination is examined. Lastly the tissue specific promotor

activation of cellular death genes may also prove to be useful in this area.

5 **Gene Therapy Chimeras:** The establishment of "chimeric DNA" for gene therapy requires the joining of different segments of DNA together to make a new DNA that has characteristics of both precursor DNA species involved in the linkage. In this proposal the two pieces being
10 linked involve different functional aspects of DNA, the promotor region which allows for the reading of the DNA for the formation of mRNA will provide specificity and the DNA sequence coding for the mRNA will provide for therapeutic functional DNA.

15 **DNA-Specified Enzyme or Cytokine mRNA:** When effective, antitumor drugs can cause the regression of very large amounts of tumor. The main requirements for antitumor drug activity is the requirement to achieve both a long enough time (t) and high enough concentration (c) (cxt)
20 of exposure of the tumor to the toxic drug to assure sufficient cell damage for cell death to occur. The drug also must be "active" and the toxicity for the tumor greater than for the hosts normal cells. The availability of the drug to the tumor depends on tumor
25 blood flow and the drugs diffusion ability. Blood flow to the tumor does not provide for selectivity as blood flow to many normal tissues is often as great or greater than that to the tumor. The majority of chemotherapeutic cytotoxic drugs are often as toxic to
30 normal tissue as to tumor tissue. Dividing cells are often more sensitive than non-dividing normal cells, but in many slow growing solid tumors such as prostatic cancer this does not provide for antitumor specificity.

35 Previously a means to increase tumor specificity of antitumor drugs was to utilize tumor associated enzymes to activate nontoxic prodrugs to cytotoxic agents. A

problem with this approach was that most of the enzymes found in tumors were not totally specific in their activity and similar substrate active enzymes or the same enzyme at only slightly lower amounts was found in
5 other tissue and thus normal tissues were still at risk for damage.

To provide absolute specificity and unique activity, viral, bacterial and fungal enzymes which have unique
10 specificity for selected prodrugs were found which were not present in human or other animal cells. Attempts to utilize enzymes such as herpes simplex thymidine kinase, bacterial cytosine deaminase and carboxypeptidase G-2 were linked to antibody targeting
15 systems with modest success. Unfortunately, antibody targeted enzymes limit the number of enzymes available per cell. Also, most antibodies do not have a high tumor target to normal tissue ratio thus normal tissues are still exposed reducing the specificity of these
20 unique enzymes. Antibodies are large molecules that have poor diffusion properties and the addition of the enzymes molecular weight further reduces the antibodies diffusion.

25 Gene therapy could produce the best desired result if it could achieve the specific expression of a protein in the tumor and not normal tissue in order that a high local concentration of the enzyme be available for the production in the tumor environment of active drug.

30

Cytokines:

Results demonstrated that tumors such as the bladder and prostate were not immunogenic, that is the administration of irradiated tumor cells to the animal
35 prior to subsequent administration of non-irradiated tumor cells did not result in a reduction of either the number of tumor cells to produce a tumor nor did it

reduce the growth rate of the tumor. But if the tumor was transfected with a retrovirus and secreted large concentrations of cytokines such as IL-2 then this could act as an antitumor vaccine and could also reduce the growth potential of an already established and growing tumor. IL-2 was the best, GM-CSF also had activity whereas a number of other cytokines were much less active. In clinical studies just using IL-2 for immunostimulation, very large concentrations had to be given which proved to be toxic. The key to the success of the cytokine gene modified tumor cell is that the cytokine is produced at the tumor site locally and is not toxic and that it stimulates immune recognition of the tumor and allows specific and non toxic recognition and destruction of the tumor. The exact mechanisms of how IL-2 production by the tumor cell activates immune recognition is not fully understood, but one explanation is that it bypasses the need for cytokine production by helper T cells and directly stimulates tumor antigen activated cytotoxic CD8 cells. Activation of antigen presenting cells may also occur.

Tissue Promotor-Specific Chimera DNA Activation

25 Non-Prostatic Tumor Systems:

It has been observed in non-prostatic tumors that the use of promotor specific activation can selectively lead to tissue specific gene expression of the transfected gene. In melanoma the use of the tyrosinase promotor which codes for the enzyme responsible for melanin expression produced over a 50 fold greater expression of the promotor driven reporter gene expression in melanoma cells and not non melanoma cells. Similar specific activation was seen in the melanoma cells transfected when they were growing in mice. In that experiment no non-melanoma or melanocyte cell expressed the tyrosinase drive reporter gene

product. The research group at Wellcome Laboratories have cloned and sequenced the promoter region of the gene coding for carcinoembryonic antigen (CEA). CEA is expressed on colon and colon carcinoma cells but specifically on metastatic. A gene chimera was generated which cytosine deaminase. Cytosine deaminase which converts 5 fluororocytosine into 5 fluorouracil and observed a large increase in the ability to selectively kill CEA promotor driven colon tumor cells but not normal liver cells. In vivo they observed that bystander tumor cells which were not transfected with the cytosine deaminase gene were also killed, and that there was no toxicity to the host animal as the large tumors were regressing following treatment. Herpes simplex virus, (HSV), thymidine kinase similarly activates the prodrug gancyclovir to be toxic towards dividing cancer cells and HSV thymidine kinase has been shown to be specifically activatable by tissue specific promoters.

20
Prostatic Tumor Systems: The therapeutic key to effective cancer therapy is to achieve specificity and spare the patient toxicity. Gene therapy may provide a key part to specificity in that non-essential tissues such as the prostate and prostatic tumors produce tissue specific proteins, such as acid phosphatase (PAP), prostate specific antigen (PSA), and a gene which was cloned, prostate-specific membrane antigen (PSM). Tissues such as the prostate contain selected tissue specific transcription factors which are responsible for binding to the promoter region of the DNA of these tissue specific mRNA. The promoter for PSA has been cloned. Usually patients who are being treated for metastatic prostatic cancer have been put on androgen deprivation therapy which dramatically reduces the expression of mRNA for PSA. PSM on the other hand increases in expression with hormone

deprivation which means it would be even more intensely expressed on patients being treated with hormone therapy.

5 EXAMPLE 3:

CLONING AND CHARACTERIZATION OF THE PROSTATE SPECIFIC
MEMBRANE ANTIGEN (PSM) PROMOTER.

10

The expression and regulation of the PSM gene is complex. By immunostaining, PSM antigen was found to be expressed brilliantly in metastasized tumor, and in organ confined tumor, less so in normal prostatic tissue and more heterogenous in BPH. PSM is strongly
15 expressed in both anaplastic and hormone refractory tumors. PSM mRNA has been shown to be down regulated by androgen. Expression of PSM RNA is also modulated by a host of cytokines and growth factors. Knowledge of
20 the regulation of PSM expression should aid in such diagnostic and therapeutic strategies as immunoscintigraphic imaging of prostate cancer and prostate-specific promoter-driven gene therapy.

25 Sequencing of a 3 kb genomic DNA clone revealed that two stretches of about 300 B.P. (-260 to -600; and -1325 to -1625) have substantial homology (79-87%) to known genes. The promoter lacks a GC rich region, nor does it have a consensus TATA box. However, it
30 contains a TA-rich region from position -35 to -65.

Several consensus recognition sites for general transcription factors such as AP1, AP2, NFkB, GRE and E2-RE were identified. Chimeric constructs containing
35 fragments of the upstream region of the PSM gene fused to a promoterless chloramphenicol acetyl transferase gene were transfected into, and transiently expressed

in LNCaP, PC-3, and SW620 (a colonic cell line). With an additional SV40 enhancer, sequence from -565 to +76 exhibited promoter activity in LNCaP but not in PC-3 nor in SW620.

5

Materials and Methods

Cell Lines. LNCaP and PC-3 prostatic carcinoma cell lines (American Type Culture Collection) were cultured in RPMI and MEM respectively, supplemented with 5% fetal calf serum at 37°C and 5% CO₂. SW620, a colonic cell line.

Polymerase Chain Reaction. The reaction was performed in a 50 l volume with a final concentration of the following reagents: 16.6 mM NH₄SO₄, 67 mM Tris-HCl pH 8.8, acetylated BSA 0.2 mg/ml, 2mM MgCl₂, 250µM dNTPs, 10 mM β-mercaptoethanol, and 1 U of the 111 Taq polymerase (Boehringer Mannheim, CA). A total of 25 cycles were completed with the following profile: cycle 1, 94°C 4 min.; cycle 2 through 25, 94°C 1 min, 60°C 1 min, 72°C 1 min. The final reaction was extended for 10 min at 72°C. Aliquots of the reaction were electrophoresed on 1 % agarose gels in 1X Tris-acetate-EDTA buffer.

Cloning of PSM promoter. A bacteriophage P1 library of human fibroblast genomic DNA (Genomic Systems, Inc., St. Louis, MI), was screened using a PCR method of Pierce et al. Primers located at the 5' end of PSM cDNA were used: 5'-CTCAAAAGGGGCCGATTTC-3' and 5'-CTCTCAATCTCACTAATGCCTC-3'. A positive clone, p683, was digested with XhoI restriction enzyme. Southern analysis of the restricted fragments using a DNA probe from the extreme 5' to the Ava-I site of PSM cDNA confirmed that a 3Kb fragment contains the 5'

regulatory sequence of the PSM gene. The 3 kb XhoI fragment was subcloned into pKSBluescript vectors and sequenced using the dideoxy method.

5 **Functional Assay of PSM Promoter.** Chloramphenicol Acetyl Transferase, (CAT) gene plasmids were constructed from the SmaI-HindIII fragments or subfragments (using either restriction enzyme subfragments or PCR) by insertion into promoterless
10 pCAT basic or pCAT-enhancer vectors (Promega). pCAT-constructs were cotransfected with pSV β gal plasmid (5 μ g of each plasmid) into cell lines in duplicates, using a calcium phosphate method (Gibco-BRL, Gaithersburg, MD). The transfected cells were
15 harvested 72 hours later and assayed (15 μ g of lysate) for CAT activity using the LSC method and for β gal activity (Promega). CAT activities were standardized by comparison to that of the β gal activities.

20 **Results**

Sequence of the 5' end of the PSM gene.

The DNA sequence of the 3 kb XhoI fragment of p683 which includes 3017 bp of DNA from the RNA start site
25 was determined. (Figure 15) The sequence from the XhoI fragment displayed a remarkable arrays of elements and motifs which are characteristic of eukaryotic promoters and regulatory regions found in other genes (Figure 16).

30

Functional Analysis of upstream PSM genomic elements for promoter activity.

Various pCAT-PSM promoter constructs were tested for
35 promoter activities in two prostatic cell lines: LNCaP, PC-3 and a colonic SW620 (Figure 17). Induction of CAT activity was neither observed in p1070-CAT which

contained a 1070 bp PSM 5' promoter fragment, nor in p676-CAT which contained a 641 bp PSM 5' promoter fragment. However, with an additional SV-40 enhancer, sequence from -641 to -1 (p676-CATE) exhibited promoter activity in LNCaP but not in PC-3 nor in SW620.

Therefore, a LNCaP specific promoter fragment from -641 to -1 has been isolated which can be used in PSM promoter-driven gene therapy.

EXAMPLE 4:

ALTERNATIVELY SPLICED VARIANTS OF PROSTATE SPECIFIC MEMBRANE ANTIGEN RNA: RATIO OF EXPRESSION AS A POTENTIAL MEASUREMENT OF PROGRESSION

MATERIALS AND METHODS

Cell Lines. LNCaP and PC-3 prostatic carcinoma cell lines were cultured in RPMI and MEM respectively, supplemented with 5% fetal calf serum at 37°C and 5% CO₂.

Primary tissues. Primary prostatic tissues were obtained from MSKCC's in-house tumor procurement service. Gross specimen were pathologically staged by MSKCC's pathology service.

RNA Isolation. Total RNA was isolated by a modified guanidinium thiocyanate/phenol/chloroform method using a RNazol B kit (Tel-Test, Friendswood, TX). RNA was stored in diethyl pyrocarbonate-treated water at -80°C. RNA was quantified using spectrophometric absorption at 260nm.

cDNA synthesis. Two different batches of normal prostate mRNAs obtained from trauma-dead males

(Clontech, Palo Alto, CA) were denatured at 70°C for 10 min., then reverse transcribed into cDNA using random hexamers and Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) at 50°C for 30 min.
5 followed by a 94°C incubation for 5 min.

Polymerase Chain Reaction. Oligonucleotide primers (5'-CTCAAAGGGGCCGGATTTC-3' and 5'-AGGCTACTTCACTCAAAG-3'), specific for the 5' and 3' ends
10 of PSM cDNA were designed to span the cDNA sequence. The reaction was performed in a 50 µl volume with a final concentration of the following reagents: 16.6 mM NH₄SO₄, 67 mM Tris-HCl pH 8.8, acetylated BSA 0.2 mg/ml, 2mM MgCl₂, 250µM dNTPs, 10 mM β-mercaptoethanol, and 1
15 U of rTth polymerase (Perkin Elmer, Norwalk, CT). A total of 25 cycles were completed with the following profile: cycle 1, 94°C 4 min.; cycle 2 through 25, 94°C 1 min, 60°C 1 min, 72°C 1 min. The final reaction was extended for 10 min at 72°C. Aliquots of the reaction
20 were electrophoresed on 1 % agarose gels in 1X Tris-acetate-EDTA buffer.

Cloning of PCR products. PCR products were cloned by the TA cloning method into pCRII vector using a kit
25 from Invitrogen (San Diego, CA). Ligation mixture were transformed into competent *Escherichia coli* Inv5α.

Sequencing. Sequencing was done by the dideoxy method using a sequenase kit from US Biochemical
30 (Cleveland, OH). Sequencing products were electrophoresed on a 5% polyacrylamide/7M urea gel at 52°C.

RNase Protection Assays. Full length PSM cDNA clone
35 was digested with NgoM I and NheI. A 350 b.p. fragment was isolated and subcloned into pSPORT1 vector (GIBCO-BRL, Gaithersburg, MD). The resultant plasmid, pSP350,

was linearized, and the insert was transcribed by SP6 RNA polymerase to yield antisense probe of 395 nucleotide long, of which 355 nucleotides and/or 210 nucleotides should be protected from RNase digestion by PSM RNA respectively. Total cellular RNA (20 µg) from different tissues were hybridized to the aforementioned antisense RNA probe. Assays were performed as described. tRNA was used as negative control. RPAs for LNCaP and PC-3 were repeated.

RESULTS

RT-PCR of mRNA from normal prostatic tissue. Two independent RT-PCR of mRNA from normal prostates were performed as described in *Materials and Methods*. Subsequent cloning and sequencing of the PCR products revealed the presence of an alternatively spliced variant, PSM'. PSM' has a shorter cDNA (2387 nucleotides) than PSM (2653 nucleotides). The results of the sequence analysis are shown in Figure 18. The cDNAs are identical except for a 266 nucleotide region near the 5' end of PSM cDNA (nucleotide 114 to 380) that is absent in PSM' cDNA. Two independent repetitions of RT-PCR of different mRNA samples yielded identical results.

RNase Protection Assays. An RNA probe complementary to PSM RNA and spanning the 3' splice junction of PSM' RNA was used to measure relative expression of PSM and PSM' mRNAs (Figure 19). With this probe, both PSM and PSM' RNAs in LNCaP cells was detected and the predominant form was PSM. Neither PSM nor PSM' RNA was detected in PC-3 cells, in agreement with previous Northern and Western blot data. Figure 20 showed the presence of both splice variants in human primary prostatic tissues. In primary prostatic tumor, PSM is the dominant form. In contrast, normal prostate expressed more PSM' than PSM. BPH samples showed about equal

expression of both variants.

Tumor Index. The relative expression of PSM and PSM' (Figure 36) was quantified by densitometry and expressed as a tumor index (Figure 21). LNCaP has an index ranging from 9-11; CaP from 3-6; BPH from 0.75 to 1.6; normal prostate has values from 0.075 to 0.45.

DISCUSSION

Sequencing data of PCR products derived from human normal prostatic mRNA with 5' and 3' end PSM oligonucleotide primers revealed a second splice variant, PSM', in addition to the previously described PSM cDNA.

PSM is a 750 a.a. protein with a calculated molecular weight of 84,330. PSM was hypothesized to be a type II integral membrane protein. A classic type II membrane protein is the transferrin receptor and indeed PSM has a region that has modest homology with the transferrin receptor. Analysis of the PSM amino acid sequence by either the methods of Rao and Argos or Eisenberg et. al. strongly predicted one transmembrane helix in the region from a.a.#20 to #43. Both programs found other regions that could be membrane associated but were not considered likely candidates for being transmembrane regions.

PSM' antigen, on the other hand, is a 693 a.a. protein as deduced from its mRNA sequence with a molecular weight of 78,000. PSM' antigen lacks the first 57 amino acids present in PSM antigen (Figure 18). It is likely that PSM' antigen is cytosolic.

The function of PSM and PSM' are probably different. The cellular location of PSM antigen suggests that it may interact with either extra- or intra- cellular

ligand(s) or both; while that of PSM' implies that PSM' can only react with cytosolic ligand(s). Furthermore, PSM antigen has 3 potential phosphorylation sites on its cytosolic domain. These sites are absent in PSM' antigen. On the other hand, PSM' antigen has 25 potential phosphorylation sites, 10 N-myristoylation sites and 9 N-glycosylation sites. For PSM antigen, all of these potential sites would be on the extracellular surface. The modifications of these sites for these homologous proteins would be different depending on their cellular locations. Consequently, the function(s) of each form would depend on how they are modified.

The relative differences in expression of PSM and PSM' by RNase protection assays was analyzed. Results of expression of PSM and PSM' in primary prostatic tissues strongly suggested a relationship between the relative expression of these variants and the status of the cell: either normal or cancerous. While it is noted here that the sample size of the study is small (Figures 20 and 21), the consistency of the trend is evident. The samples used were gross specimens from patients. The results may have been even more dramatic if specimens that were pure in content of CaP, BPH or normal had been used. Nevertheless, in these specimens, it is clear that there is a relative increase of PSM over PSM' mRNA in the change from normal to CaP. The Tumor Index (Figure 21) could be useful in measuring the pathologic state of a given sample. It is also possible that the change in expression of PSM over PSM' may be a reason for tumor progression. A more differentiated tumor state may be restored by PSM' either by transfection or by the use of differentiation agents.

EXAMPLE 5:

ENHANCED DETECTION OF PROSTATIC HEMATOGENOUS MICRO-METASTASES WITH PSM PRIMERS AS COMPARED TO PSA PRIMERS USING A SENSITIVE NESTED REVERSE TRANSCRIPTASE-PCR ASSAY.

77 randomly selected samples were analyzed from patients with prostate cancer and reveals that PSM and PSA primers detected circulating prostate cells in 48 (62.3%) and 7 (9.1%) patients, respectively. In treated stage D disease patients, PSM primers detected cells in 16 of 24 (66.7%), while PSA primers detected cells in 6 of 24 patients (25%). In hormone-refractory prostate cancer (stage D3), 6 of 7 patients were positive with both PSA and PSM primers. All six of these patients died within 2-6 months of their assay, despite aggressive cytotoxic chemotherapy, in contrast to the single patient that tested negatively in this group and is alive 15 months after his assay, suggesting that PSA-PCR positivity may serve as a predictor of early mortality. In post-radical prostatectomy patients with negative serum PSA values, PSM primers detected metastases in 21 of 31 patients (67.7%), while PSA primers detected cells in only 1 of 33 (3.0%), indicating that micrometastatic spread may be a relatively early event in prostate cancer. The analysis of 40 individuals without known prostate cancer provides evidence that this assay is highly specific and suggests that PSM expression may predict the development of cancer in patients without clinically apparent prostate cancer. Using PSM primers, micrometastases were detected in 4 of 40 controls, two of whom had known BPH by prostate biopsy and were later found to have previously undetected prostate cancer following repeat prostate biopsy performed for a rising serum PSA value. These results

show the clinical significance of detection of hematogenous micrometastatic prostate cells using PSM primers and potential applications of this molecular assay.

5

EXAMPLE 6:

MODULATION OF PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSM) EXPRESSION IN VITRO BY CYTOKINES AND GROWTH FACTORS.

10

The effectiveness of CYT-356 imaging is enhanced by manipulating expression of PSM. PSM mRNA expression is downregulated by steroids. This is consistent with the clinical observations that PSM is strongly expressed in both anaplastic and hormone refractory lesions. In contrast, PSA expression is decreased following hormone withdrawal. In hormone refractory disease, it is believed that tumor cells may produce both growth factors and receptors, thus establishing an autocrine loop that permits the cells to overcome normal growth constraints. Many prostate tumor epithelial cells express both TGF α and its receptor, epidermal growth factor receptor. Results indicate that the effects of TGF α and other selected growth factors and cytokines on the expression of PSM in-vitro, in the human prostatic carcinoma cell line LNCaP.

25

2x10⁶ LNCaP cells growing in androgen-depleted media were treated for 24 to 72 hours with EGF, TGF α , TNF β or TNF α in concentrations ranging from 0.1 ng/ml to 100 ng/ml. Total RNA was extracted from the cells and PSM mRNA expression was quantitated by Northern blot analysis and laser densitometry. Both b-FGF and TGF α yielded a dose-dependent 10-fold upregulation of PSM expression, and EGF a 5-fold upregulation, compared to untreated LNCaP. In contrast, other groups have shown a marked downregulation in PSA expression induced by

30

35

these growth factors in this same in-vitro model. $TNF\alpha$, which is cytotoxic to LNCaP cells, and $TNF\beta$ downregulated PSM expression 8-fold in androgen depleted LNCaP cells.

5

TGF α is mitogenic for aggressive prostate cancer cells. There are multiple forms of PSM and only the membrane form is found in association with tumor progression. The ability to manipulate PSM expression by treatment with cytokines and growth factors may enhance the efficacy of Cytogen 356 imaging, and therapeutic targeting of prostatic metastases.

10

EXAMPLE 7:

15

NEOADJUVANT ANDROGEN-DEPRIVATION THERAPY (ADT) PRIOR TO RADICAL PROSTATECTOMY RESULTS IN A SIGNIFICANTLY DECREASED INCIDENCE OF RESIDUAL MICROMETASTATIC DISEASE AS DETECTED BY NESTED RT-PCT WITH PRIMERS.

20

Radical prostatectomy for clinically localized prostate cancer is considered by many the "gold standard" treatment. Advances over the past decade have served to decrease morbidity dramatically. Improvements intended to assist clinicians in better staging patients preoperatively have been developed, however the incidence of extra-prostatic spread still exceeds 50%, as reported in numerous studies. A phase III prospective randomized clinical study designed to compare the effects of ADT for 3 months in patients undergoing radical prostatectomy with similarly matched controls receiving surgery alone was conducted. The previously completed phase II study revealed a 10% margin positive rate in the ADT group (N=69) as compared to a 33% positive rate (N=72) in the surgery alone group.

25

30

35

Patients who have completed the phase III study were analyzed to determine if there are any differences between the two groups with respect to residual micrometastatic disease. A positive PCR result in a
5 post-prostatectomy patient identifies viable metastatic cells in the circulation.

Nested RT-PCR was performed with PSM primers on 12 patients from the ADT group and on 10 patients from the
10 control group. Micrometastatic cells were detected in 9/10 patients (90%) in the control group, as compared to only 2/12 (16.7%) in the ADT group. In the ADT group, 1 of 7 patients with organ-confined disease tested positively, as compared to 3 of 3 patients in
15 the control group. In patients with extra-prostatic disease, 1 of 5 were positive in the ADT group, as compared to 6 of 7 in the control group. These results indicate that a significantly higher number of patients may be rendered tumor-free, and potentially "cured" by
20 the use of neoadjuvant ADT.

EXAMPLE 8:

SENSITIVE NESTED RT-PCR DETECTION OF CIRCULATION
25 PROSTATIC TUMOR CELLS - COMPARISON OF PSM AND PSA-BASED
ASSAYS

Despite the improved and expanded arsenal of modalities available to clinician today, including sensitive serum
30 PSA assays, CT scan, transrectal ultrasonography, endorectal co.I MRI, etc., many patients are still found to have metastatic disease at the time of pelvic lymph node dissection and radical prostatectomy. A highly sensitive reverse transcription PCR assay
35 capable of detecting occult hematogenous micrometastatic prostatic cells that would otherwise go undetected by presently available staging modalities

was developed. This assay is a modification of similar PCR assays performed in patients with prostate cancer and other malignancies. The assay employs PCR primers derived from the cDNA sequences of prostate-specific antigen⁶ and the prostate-specific membrane antigen recently cloned and sequenced.

Materials and Methods

10 **Cells and Reagents.** LNCaP and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD.). Details regarding the establishment and characteristics of these cell. Cells grown in RPMI 1640 medium and supplemented with L-glutamine, 15 nonessential amino acids, and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD.) In a 5% CO₂ incubator at 37°C. All cell media was obtained from the MSKCC Media Preparation Facility. Routine chemical reagents were of the highest grade possible and were obtained 20 from Sigma Chemical Company (St. Louis, MO).

25 **Patient Blood Specimens.** All blood specimens used in this study were from patients seen in the outpatient offices of urologists on staff at MSKCC. Two anti-coagulated tubes per patient were obtained at the time of their regularly scheduled blood draws. Specimens were obtained with informed consent of each patient , as per a protocol approved by the MSKCC Institutional Review Board. Samples were promptly brought to the 30 laboratory for immediate processing. Seventy-seven specimens from patients with prostate cancer were randomly selected and delivered to the laboratory "blinded" along with samples from negative controls for processing. These included 24 patients with stage D 35 disease (3 with D₀, 3 with D¹, 11 with D², and 7 with D³), 31 patients who had previously undergone radical prostatectomy and had undetectable postoperative serum

PSA levels (18 with pT2 lesions, 11 with pT3, and 2 pT4), 2 patients with locally recurrent disease following radical prostatectomy, 4 patients who had received either external beam radiation therapy or
5 interstitial ^{125}I implants, 10 patients with untreated clinical stage T1-T2 disease, and 6 patients with clinical stage T3 disease on anti-androgen therapy. The forty blood specimens used as negative controls were from 10 health males, 9 males with biopsy-proven
10 BPH and elevated serum PSA levels, 7 healthy females, 4 male patients with renal cell carcinoma, 2 patients with prostatic intraepithelial neoplasia (PIN), 2 patients with transitional cell carcinoma of the bladder and a pathologically normal prostate, 1 patient
15 with acute prostatitis, 1 patient with acute promyelocytic leukemia, 1 patient with testicular cancer, 1 female patient with renal cell carcinoma, 1 patient with lung cancer, and 1 patient with a cyst of the testicle.

20
Blood Sample Processing/RNA Extraction. 4 ml of whole anticoagulated venous blood was mixed with 3 ml of ice cold PBS and then carefully layered atop 8 ml of Ficoll (Pharmacia, Uppsala, Sweden) in a 14-ml polystyrene
25 tube. Tubes were centrifuged at 200 x g for 30 min. at 4°C. The buffy coat layer (approx. 1 ml.) was carefully removed and rediluted to 50 ml with ice cold PBS in a 50 ml polypropylene tube. This tube was then centrifuged at 2000 x g for 30 min. at 4°C. The
30 supernatant was carefully decanted and the pellet was allowed to drip dry. One ml of RNazol B was then added to the pellet and total RNA was isolated as per manufacturers directions (Cinna/Biotechx, Houston, TX.) RNA concentrations and purity were determined by UV
35 spectroscopy on a Beckman DU 640 spectrophotometer and by gel analysis.

Determination of PCR Sensitivity. RNA was isolated from LNCaP cells and from mixtures of LNCaP and MCF-7 cells at fixed ratios (i.e. 1:100, 1:1,000, etc.) using RNazol B. Nested PCR was then performed as described
5 below with both PSA and PSM primers in order to determine the limit of detection for the assay. LNCaP:MCF-7 (1:100,000) cDNA was diluted with distilled water to obtain concentrations of 1:1,000,000. The human breast cancer cell line MCF-7 was chosen because
10 they had previously been tested by us and shown not to express either PSM nor PSA by both immunohistochemistry and conventional and nested PCR.

Polymerase Chain Reaction. The PSA outer primer
15 sequences are nucleotides 494-513 (sense) in exon 4 and nucleotides 960-979 (anti-sense) in exon 5 of the PSA cDNA. These primers yield a 486 bp PCR product from PSA CDNA that can be distinguished from a product synthesized from possible contaminating genomic DNA.

20 PSA-494 5'-TAC CCA CTG CAT CAG GAA CA-3'

PSA-960 5'-CCT TGA AGC ACA CCA TTA CA-3'

The PSA inner upstream primer begins at nucleotide 559 and the downstream primer at nucleotide 894 to yield a 355 bp PCR product.

25 PSA-559 5'-ACA CAG GCC AGG TAT TTC AG-3'

PSA-894 5'-GTC CAG CGT CCA GCA CAC AG-3'

All primers were synthesized by the MSKCC Microchemistry Core Facility. 5µg of total RNA was reverse-transcribed into cDNA using random hexamer
30 primers (Gibco-BRL) and Superscript II reverse transcriptase (Gibco-BRL) according to the manufacturers recommendations. 1µl of this CDNA served as the starting template for the outer primer PCR reaction. The 20µl PCR mix included: 0.5U Taq
35 polymerase (Promega) Promega reaction buffer, 1.5mM MgCl₂, 200µM dNTPs, and 1.0µM of each primer. This mix was then transferred to a Perkin Elmer 9600 DNA thermal

cycler and incubated for 25 cycles. The PCR profile was as follows: 94°C x 15 sec., 60°C x 15 sec., and 72°C for 45 sec. After 25 cycles, samples were placed on ice, and 1µl of this reaction mix served as the template for another 25 cycles using the inner primers. The first set of tubes were returned to the thermal cycler for 25 additional cycles. The PSM outer upstream primer sequences are nucleotides 1368-1390 and the downstream primers are nucleotides 1995-2015, yielding a 67 bp PCR product.

PSM-1368 5'-CAG ATA TGT CAT TCT GGG AGG TC-3'

PSM-2015 5'-AAC ACC ATC CCT CCT CGA ACC-3'

The PSM inner upstream primer span nucleotides 1689-1713 and the downstream primer span nucleotides 1899-1923, yielding a 234 bp PCR product.

PSM-1689 5'-CCT AAC AAA AGA GCT GAA AAG CCC-3'

PSM-1923 5'-ACT GTG ATA CAG TGG ATA GCC GCT-3'

2µl of cDNA was used as the starting DNA template in the PCR assay. The 50µl PCR mix included: 1U Taq polymerase (Boehringer Mannheim), 250µM cNTPs, 10mM β-mercaptoethanol, 2mM MgCl₂, and 5µl of a 10x buffer mix containing: 166mM NH₄SO₄, 670mM Tris pH 8.8, and 2mg/ml of acetylated BSA. PCR was carried out in a Perkin Elmer 480 DNA thermal cycler with the following parameters: 94°C x 4 minutes for 1 cycle, 94°C x 30 sec., 58°C x 1 minute, and 72°C x 1 minute for 25 cycles, followed by 72°C x 10 minutes. Samples were then iced and 2.5µl of this reaction mix was used as the template for another 25 cycles with a new reaction mix containing the inner PSM primers. cDNA quality was verified by performing control reactions using primers derived from the β-2-microglobulin gene sequence¹⁰ a ubiquitous housekeeping gene. These primers span exons 2-4 and generate a 620 bp PCR product. The sequences for these primers are:

β-2 (exon 2) 5'-AGC AGA GAA TGG AAA GTC AAA-3'

β-2 (exon 4) 5'-TGT TGA TGT TGG ATA AGA GAA-3'

The entire PSA mix and 7-10μl of each PSM reaction mix were run on 1.5-2% agarose gels, stained with ethidium bromide and photographed in an Eagle Eye Video Imaging System (Statagene, Torrey Pines, CA.). Assays were repeated at least twice to verify results.

Cloning and Sequencing of PCR Products. PCR products were cloned into the pCR II plasmid vector using the TA cloning system (Invitrogen). These plasmids were transformed into competent E. coli cells using standard methods¹¹ and plasmid DNA was isolated using Magic Minipreps (Promega) and screened by restriction analysis. Double-stranded TA clones were then sequenced by the dideoxy method using ³⁵S-cCTP (NEN) and Sequenase (U.S. Biochemical). Sequencing products were then analyzed on 6% polyacrilamide/7M urea gels, which were fixed, dried, and autoradiographed as described.

Southern Analysis. PCR products were transferred from ethidium-stained agarose gels to Nytran nylon membranes (Schleicher and Schuell) by pressure blotting with a Posi-blotter (Stratagene) according to the manufacturer's instructions. DNA was cross-linked to the membrane using a UV Stratalinker (Stratagene). Blots were pre-hybridized at 65°C for 2 hours and subsequently hybridized with denatured ³²P-labeled, random-primed cDNA probes (either PSA or PSM).^{6,7} Blots were washed twice in 1x SSC/0.5% SDS at 42°C and twice in 0.1x SSC/0.1% SDS at 50°C for 20 minutes each. Membranes were air-dried and autoradiographed for 1-3 hours at room temperature with Hyperfilm MP (Amersham).

Results

35

PSA and PSM Nested PCR Assays: The application of nested PCR increased the level of detection from an

average of 1:10,000 using outer primers alone, to better than 1:1,000,000. Dilution curves demonstrating this added sensitivity are shown for PSA and PSM-PCR in Figures 1 and 2 respectively. Figure 1 shows that the
5 486 bp product of the PSA outer primer set is clearly detectable with ethidium staining to 1:10,000 dilutions, whereas the PSA inner primer 355 bp product is clearly detectable in all dilutions shown. In Figure 2 the PSM outer primer 647 bp product is also
10 clearly detectable in dilutions to only 1:10,000 with conventional PCR, in contrast to the PSM inner nested PCR 234 bp product which is detected in dilutions as low as 1:1,000,000. Southern blotting was performed on all controls and most of the patient samples in order
15 to confirm specificity. Southern blots of the respective dilution curves confirmed the primer specificities but did not reveal any significantly increased sensitivity.

20 **PCR in Negative Controls:** Nested PSA and PSM PCR was performed on 40 samples from patients and volunteers as described in the methods and materials section. Figure 48 reveals results from 4 representative negative control specimens, in addition to a positive control.
25 Each specimen in the study was also assayed with the β -2-microglobulin control, as shown in the figure, in order to verify RNA integrity. Negative results were obtained on 39 of these samples using the PSA primers, however PSM nested PCR yielded 4 positive results. Two
30 of these "false positives" represented patients with elevated serum PSA values and an enlarged prostate who underwent a transrectal prostate biopsy revealing stromal and fibromuscular hyperplasia. In both of these patients the serum PSA level continued to rise
35 and a repeat prostate biopsy performed at a later date revealed prostate cancer. One patient who presented to the clinic with a testicular cyst was noted to have a

positive PSM nested PCR result which has been unable to explain. Unfortunately, this patient never returned for follow up, and thus have not been able to obtain another blood sample to repeat this assay. Positive
5 result were obtained with both PSA and PSM primers in a 61 year old male patient with renal cell carcinoma. This patient has a normal serum PSA level and a normal digital rectal examination. Overall, if the two
10 patients were excluded in whom a positive PCR, but no other clinical test, accurately predicted the presence of prostate cancer, 36/38 (94.7%) of the negative controls were negative with PSM primers, and 39/40 (97.5%) were negative using PSA primers.

15 **Patient Samples:** In a "blinded" fashion, in which the laboratory staff were unaware of the nature of each specimen, 117 samples from 77 patients mixed randomly with 40 negative controls were assayed. The patient
20 samples represented a diverse and heterogeneous group as described earlier. Several representative patient samples are displayed in Figure 49, corresponding to positive results from patients with both localized and disseminated disease. Patients 4 and 5, both with
25 stage D prostate cancer exhibit positive results with both the outer and inner primer pairs, indicating a large circulating tumor cell burden, as compared to the other samples. Although the PSM and PSA primers yielded similar sensitivities in LNCaP dilution curves
30 as previously shown, PSM primers detected micrometastases in 62.3% of the patient samples, whereas PSA primers only detected 9.1%. In patients with documented metastatic prostate cancer (stages D₀ - D₃) receiving anti-androgen treatment, PSM primers
35 detected micrometastases in 16/24 (66.7%), whereas PSA primers detected circulating cells in only 6/24 (25%). In the study 6/7 patients with hormone-refractory prostate cancer (stage D₃) were positive. In the

study, PSA primers revealed micrometastatic cells in only 1/15 (6.7%) patients with either pT3 or pT4 (locally-advanced) prostate cancer following radical prostatectomy. PSM primers detected circulating cells in 9/15 (60%) of these patients. Interestingly, circulating cells 13/18 (72.2%) patients with pT2 (organ-confined) prostate cancer following radical prostatectomy using PSM primers was detected. None of these patient samples were positive by PSA-PCR.

10

Improved and more sensitive method for the detection of minimal, occult micrometastatic disease have been reported for a number of malignancies by use of immunohistochemical methods, as well as the polymerase chain reaction. The application of PCR to detect occult hematogenous micrometastases in prostate cancer was first described by Moreno, et al. using conventional PCR with PSA-derived primers.

20 When human prostate tumors and prostate cancer cells in-vitro were studied by immunohistochemistry and mRNA analysis, PSM appeared to be highly expressed in anaplastic cells, hormone-refractory cells, and bony metastases, in contrast to PSA. If cells capable of hematogenous micrometastasis represent the more aggressive and poorly-differentiated cells, they may express a higher level of PSM per cell as compared to PSA, enhancing their detectability by RT-PCR.

30 Nested RT-PCR assays are both sensitive and specific. Results have been reliably reproduced on repeated occasions. Long term testing of both cDNA and RNA stability is presently underway. Both assays are capable of detecting one prostatic cell in at least one million non-prostatic cells of similar size. This confirms the validity of the comparison of PSM vs. PSA primers. Similar levels of PSM expression in both

35

human prostatic cancer cells in-vivo and LNCaP cells in-vitro resulted. The specificity of the PSM-PCR assay was supported by the finding that two "negative control" patients with positive PSM-PCR results were both subsequently found to have prostate cancer. This suggests an exciting potential application for this technique for use in cancer screening. In contrast to recently published data, significant ability for PSA primers to accurately detect micrometastatic cells in patients with pathologically with pathologically organ-confined prostate cancer, despite the sensitivity of the assay failed to result. Rather a surprisingly high percentage of patients with localized prostate cancer that harbor occult circulating prostate cells following "curative" radical prostatectomy results which suggests that micrometastasis is an early event in prostate cancer.

The application of this powerful new modality to potentially stage and/or follow the response to therapy in patients with prostate cancer certainly merits further investigation. In comparison to molecular detection of occult tumor cells, present clinical modalities for the detection of prostate cancer spread appear inadequate.

Transition of prostate cancer from androgen dependent to androgen independent state is a clinically important step which may be caused or accompanied by genetic changes. Expression of prostate specific membrane antigen (PSM) is most intense in LNCaP cells, an androgen dependent prostate carcinoma cell line: and is not detectable in PC-3 nor in DU-145 cells, which are androgen independent prostate carcinoma cell lines. A microsatellite repeat of (TTTGG), (TTTG), has been found in the first intron of the PSM gene. Our hypothesis is that this Microsatellite repeat could be

a cis-acting element in the regulation of PSM expression. A polymeric chain reaction amplifying this repeat was used to look for any gene alteration in several cell lines: LNCap, PC-3, PC-3M, DU-145 as well
5 as in 20 paired normal and early prostatic cancers (p12-4, NO). In addition, immunohistochemistry (IHC) was used to analyze PSM expression in patient samples. By IHC, no detectable expression in DU-145, PC-3, and PC-3M was found, but all tumor expressed PSM. Further
10 sequencing data of the microsatellite repeat confirmed no change in LNCap, and in contrast, an amplification in PC-3 and a gross deletion in DU-145. Alteration of a T segment adjacent to the microsatellite repeat was found in one tumor sample. These results suggest that
15 there is rarely alteration in the intronic microsatellite sequence of the PSM gene in early prostate cancer. The abnormal pattern in the absence of expression suggest genetic instability in the more aggressive tumor lines such as the PC-3, PC-3M and DU-
20 145 cells.

EXAMPLE 9:

CHROMOSOMAL LOCALIZATION OF COSMID CLONES 194 AND 683
5 BY FLUORESCENCE IN-SITU HYBRIDIZATION:

PSM was initially mapped as being located on chromosome
11p11.2-p13 (Figures 25-27). Further information from
the cDNA in-situ hybridizations experiments
10 demonstrated as much hybridization on the q as p arms.
Much larger fragments of genomic DNA was obtained as
cosmids and two of these of about 60 kilobases each one
going 3' and the other 5' both demonstrated binding to
chromosome 11 p and q under low stringency. However
15 under higher stringency conditions only the binding at
11q14-q21 remained. This result suggests that there is
another gene on 11p that is very similar to PSM because
it is so strongly binding to nearly 120 kilobases of
genomic DNA (Figure 28).

20 Purified DNA from cosmid clones 194 and 683 was
labelled with biotin dUTP by nick translation.
Labelled probes were combined with sheared human DNA
and independently hybridized to normal metaphase
25 chromosomes derived from PHA stimulated peripheral
blood lymphocytes in a solution containing 50%
formamide, 10% dextran sulfate, and 2XSSC. Specific
hybridization signals were detected by incubating the
hybridized slides in fluorescein conjugated avidin.
30 Following signal detection the slides were
counterstained with propidium iodide and analyzed.
These first experiments resulted in the specific
labelling of a group C chromosome on both the long and
short arms. This chromosome was believed to be
35 chromosome 11 on the basis of its size and morphology.
A second set of experiments were performed in which a
chromosome 11 centromere specific probe was

- cohybridized with the cosmid clones. These experiments were carried out in 60% formamide in an attempt to eliminate the cross reactive signal which was observed when low stringency hybridizations were done. These experiments resulted in the specific labelling of the centromere and the long arm of chromosome 11. Measurements of 10 specifically labelled chromosomes 11 demonstrated that the cosmid clones are located at a position which is 44% of the distance from the centromere to the telomere of chromosome arm 11q, an area that corresponds to band 14q. A total of 160 metaphase cells were examined with 153 cells exhibiting specific labelling.
- Cloning of the 5' upstream and 3' downstream regions of the PSM genomic DNA. A bacteriophage P1 library of human fibroblast genomic DNA (Genomic Systems, St. Louis, MI) was screened using the PCR method of Pierce et. al. Primer pairs located at either the 5' or 3' termini of PSM cDNA were used. Positive cosmid clones were digested with restriction enzymes and confirmed by Southern analysis using probes which were constructed from either the 5' or 3' ends of PSM cDNA. Positive clone p683 contains the 5' region of PSM cDNA and about 60 kb upstream region. Clone -194 contains the 3' terminal of the PSM cDNA and about 60 kb downstream.

EXAMPLE 10: PEPTIDASE ENZYMATIC ACTIVITY

- Prostate Specific Membrane Antigen has activity as a carboxypeptidase and acts on both gamma linked or alpha linked amino acids which have acidic amino acids such as glutamate in the carboxy terminus.
- Prostate specific membrane antigen is found in high concentration in the seminal plasma. PSM antigen has enzymatic activity with N-acetylaspartylglutamate as a

substrate and enzymatic action results in the release of, N-acetylaspartate and glutamic acid. Because PSM action will release glutamate, and because it is well known that the seminal fluid is highly enriched in its content of glutamic acid, the action of PSM antigen of endogenous protein/peptide substrates may be responsible for generating the glutamic acid present.

It is also uncertain as to the role that seminal plasma glutamic acid plays in fertility functions. It may be that interruption of PSM antigen enzymatic activity may block the generation of glutamate and could impact on seminal plasma glutamic acid levels and its attendant fertility functions. Thus agents which inhibit PSM antigen may prove to be useful in attenuating male fertility.

EXAMPLE 11: IONOTROPIC GLUTAMATE RECEPTORS IN PROSTATE TISSUE

Prostate Specific Membrane antigen acts on N-acetylaspartylglutamic acid to release glutamate and because a homologous protein has been found in the rat brain which acts on N-acetylaspartylglutamate to free glutamate and N-acetylaspartate and because these amino acids are considered to function as neurotransmitters, the enzyme is considered to be potentially important in modulating neurotransmitter excitatory amino acid signalling as a neurocarboxypeptidase. This could be important in the prostate as well, because of the neuroendocrine nature of a subpopulation of cells in the prostate which are considered to be important synthesizing neuropeptide signaling molecules. PSM antigen from the LNCaP cell was isolated and LNCaP cells can be induced to exhibit a "neuron like" phenotype.

Excitatory neurotransmission in the central nervous system (CNS) is mediated predominantly by glutamate receptors. Two types of glutamate receptors have been identified in the human CNS: metabotropic receptors, which serve G-protein coupled second messenger signalling systems, and ionotropic receptors, which serve as ligand gated ion channels. Ionotropic glutamate channels can increase the inward flow of ions such as calcium ions. This can result in the subsequent stimulation of nitric oxide, and nitric oxide modulation of a number of signalling pathways. Nitric oxide has been found to be a major signalling mechanism involved in cell growth and death, response to inflammation, smooth muscle cell contraction etc.

Methods: Detection of glutamate receptor expression was performed using anti-gluR2/3 and anti-gluR4 polyclonal antibodies and antibiotin immunohistochemical techniques in paraffin-embedded human prostate tissues.

Results: Anti-gluR2/3 immunoreactivity was unique to prostatic stroma and was absent in the prostatic epithelial compartment. Strong anti-gluR4 immunoreactivity was observed in the basal cells of the prostate. This implied a differential location and function of glutamate receptors as defined by these antibodies.

Discussion: Distribution of glutamate receptors in the prostate has not been described. Basal cells are considered the precursor cell for the prostatic acinar and neuroendocrine cells of the prostate. Glutamate receptors may provide signalling functions in their interactions with the prostate stroma and acinar cells, and PSM may be involved in that interaction. Thus inhibition or enhancement of PSM activity could serve to modulate activity of the basal cells and prove to be

a valuable aid for controlling basal cell function in the prostate.

5 The finding of glutamate like receptors in the stroma is of interest because a large part of the prostate volume is due to stromal cells. Current observation have suggested that these stromal cells have a smooth muscle cell phenotype and thus the presence of glutamate receptors may play a role in their biologic
10 function and regulation of differentiation. A most common disease in men is the abnormal benign growth of the prostate termed benign prostatic hyperplasia, BPH.

In areas of BPH a decrease in the level of expression
15 of PSM antigen was observed. If PSM antigen activity is providing an aspect of the signalling for normal stromal function then the abnormal growth seen in BPH may be a response to that decreased activity and agents to restore its function could play a role in the
20 treatment or prevention of BPH.

Altering PSM antigen function may have beneficial actions outside the prostate. In the rat CNS a protein
25 homology to PSM antigen was discovered and provides a rational to consider prostate specific membrane antigen as a neurocarboxypeptidase. Alterations in its function may occur in neurotoxic disorders such as epilepsy, or ALS, alzheimers, and multiple sclerosis.

30

35

EXAMPLE 12: IDENTIFICATION OF A MEMBRANE-BOUND
PTEROYLPOLYGAMMAGLUTAMYL
CARBOXYPEPTIDASE (FOLATE HYDROLASE)
THAT IS EXPRESSED IN HUMAN PROSTATIC
CARCINOMA

5

As described PSM functions as a carboxypeptidase to hydrolyze both alpha and gamma peptide linkages with amino acids such as glutamate in the terminal carboxy position. The proximal small intestine (duodenum-strong expression PSM) but not the distal small intestine (ileum-absent PSM) was also very rich in expression of message for prostate specific membrane antigen in RNase protection assays. PSM antigen by immunohistochemistry was observed in the brush border membranes of the duodenum. This location was consistent with a hydrolase known as folate conjugase (folate hydrolase as a carboxypeptidase, not an endopeptidase) that had been described in the older literature, with the protein having been partially purified from the human small intestine. No cloning or sequencing of this gene had been done. There is a form of folate hydrolase that is found in all cells in the lysosomes and it was recently sequenced. There is no sequence relationship between the lysosomal endopeptidase. Membrane fraction of the LNCaP cells was very rich in folate hydrolase activity. The PSM specific monoclonal could be used to immunoprecipitate the folate hydrolase activity. This result always has the possibility that the folate hydrolase activity is not the same as PSM antigen but is a coprecipitating contaminant. Therefore PSM antigen was transfected into PC-3 cells. PC-3 cells do not express PSM nor do they have membrane folate hydrolase activity. In cells transfected with PSM antigen however expression of folate hydrolase activity was observed in the membranes. Thus PSM is a novel folate hydrolase,

10

15

20

25

30

35

folate carboxypeptidase, and is active in sequentially removing the terminal gamma-linked glutamates. In the proximal small intestine it is understandable why this enzyme would be in such a place, as the majority of
5 folate available from food is polygammglutamated and this enzyme is responsible for its hydrolysis.

Materials: Methotrexate triglutamate ($4\text{-NH}_2\text{-10-CH}_3\text{-PteGlu}_4$ (MTXglu₃)), pteroylpentaglutamate (PteGlu₅), and
10 para-aminobenzoylpentaglutamate, (pABAGlu₅) were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland) and samples were > 98% pure when evaluated by HPLC. N-acetyl- α -aspartylglutamate (NAAG) (40 Ci/mmol) was purchased from New England Nuclear
15 (Boston, MA). Protein A Sepharose 4 Fast Flow was purchased from Pharmacia (Piscataway, NJ). The 7E11-C5 monoclonal antibody to prostate specific membrane antigen was obtained from Cytogen Corporation, Princeton, NJ. All other reagents (p-
20 hydroxymercuribenzoate, homocysteine, dithiothreitol (DTT), reduced glutathione) were of the highest purity commercially available from Sigma Chemical Co. (St. Louis, MO).

25 Culture and growth of human prostate adenocarcinoma cells (LNCaP, PC-3, TSU-Pr1, and Duke-145): LNCaP cells were maintained in defined culture medium, RPMI-1640 medium supplemented with non-essential amino acids, 5 mM glutamine, and 5% heat-inactivated fetal
30 calf serum. Duke-145, PC-3, and TSU-Pr1 cells were grown in minimal essential medium (MEM), Ham's F-12K, and MEM, respectively, containing 5% fetal calf serum. No antibiotic was included in the media. Cells (1×10^6) were plated in T-75 tissue culture flasks
35 containing 15 mL of medium and incubated at 37 °C in a humidified atmosphere of 5% CO₂. Cell numbers were determined using a Model Z F Coulter Counter (Coulter

Electronic, Inc.). Prostate cells were harvested from plates by gentle scraping at 4 °C into phosphate buffered saline (136.9 mM NaCl, 2.68 mM KCl, 8.10 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.34, PBS) and centrifuged at 500 X g to obtain a cell pellet. Sedimented cells were routinely rinsed twice with 15 mL volumes of PBS.

Transfection of PSM into PC-3 Prostate Cell Line: The full length 2.65 kb PSM cDNA was subcloned into a pREP7 eukaryotic expression vector (Invitrogen, San Diego, CA) as previously described. Plasmid DNA was purified from transfected DH5- α (Gibco-BRL) using a Qiagen maxi prep plasmid isolation kit (Qiagen Inc., Chatsworth, CA). Purified plasmid DNA (5 μ g) was diluted with 300 μ L of serum free RPMI media and mixed with 45 μ L of lipofectamine (Gibco-BRL) which was previously diluted with 300 μ L of serum free RPMI media to allow an DNA-liposome complex to form. The mixture was kept at room temperature for 30 minutes, then added to a 60 mm petri dish containing 60-70% confluent PC-3 cells in 2.4 mL serum free RPMI. The DNA-liposome complex containing serum free media was mixed gently to ensure uniform distribution and was then incubated for 6 h at 37 °C in a CO₂ incubator. Following incubation, the media containing liposome-DNA complex was aspirated and replaced with 6 mL of regular growth media (10% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamine). After 48 hours, cells were trypsinized and split 1:3 into 60 mm dishes containing regular media supplemented with 200 μ g/mL of hygromycin B (Calbiochem, LaJolla, CA). Cells were maintained for 2 weeks with changes of media containing hygromycin B every third day until discrete colonies appeared. Colonies were isolated using a 6 mm cloning cylinder and were expanded in the same media. As a control, PC-3 cells were also transfected with the pREP7 vector alone.

Immunohistochemistry: The 7E11-C5 monoclonal antibody to prostate specific antigen was used. This antibody recognizes a portion of carbohydrate-containing peptide epitope on the amino terminal end of PSM that is located on the inner portion of the cytosolic membrane. After permeabilization of LNCaP and PC-3 transfected and non-transfected cells with a mixture of acetone and methanol (1:1 v/v) and blocking with 5% bovine serum albumin in 50 mM Tris buffered saline (TBS) pH 7.45, samples were incubated with 7E11-C5 antibody (20 µg/mL) for 1 h at room temperature. Negative controls were generated by substituting the same concentration of mouse IgG2ak for the PSM antibody. Using a secondary IgG, anti-mouse antibody conjugated with alkaline phosphatase, samples were re-incubated for 1 h, rinsed in TBS, and stained with bromochloroindolylphenol phosphate in 2-amino-2-methyl-1-propanol buffer. Cells expressing PSM demonstrate an intense blue staining.

Cell Membrane Preparation: Cell lysates were prepared by sonicating approximately 6×10^6 cells in 50 mM Tris pH 7.4 buffer (2 x 10 s pulses at 20 mWatts) in an ice-bath. Membrane fractions were obtained by centrifuging lysates at 100,000 x g for 30 mins. The supernatant fractions were saved and pelleted membranes were re-suspended by gentle trituration and re-sedimented at 100,000 x g for 30 mins through 10 mL of cold 50 mM Tris pH 7.4 buffer. Washed membrane fractions were dissolved in 50 mM Tris pH 7.4 buffer containing 0.1% Triton X-100 (Tris/Triton). Enzymatic activity and immunoprecipitation preparations were performed using this membrane preparation.

Immunoprecipitation of PSM from Membrane: Membrane pellets (-1 mg protein) solubilized in Tris/Triton buffer were incubated at 4 °C for 1 h in the presence of 7E11-C5 anti-prostate monoclonal antibody (6 µg

protein). Protein A Sepharose gel equilibrated in Tris/Triton buffer was added to the immunocomplex. This preparation was subsequently incubated for an additional hour at 4 °C. Sepharose beads were centrifuged at 500 x g for 5 mins and rinsed twice with Tris/Triton buffer at pH 7.4. Isolated beads were resuspended in 0.1 M glycine buffer pH 3.0, vortexed, and the supernatant fraction was assayed for hydrolase activity using MTXglu₃.

Pteroyl Gamma-Glutamyl Hydrolase Assay: Hydrolase activity was determined using capillary electrophoresis. The standard assay mixture contained 50 uM MTXGlu₃, 50 mM acetate buffer (pH 4.5) and enzyme to a final volume of 100 uL. A sample preparation without enzyme was incubated concurrently with enzymatic assays and reactions were conducted for times varying between 0 and 240 min at 37 °C. Activities were also determined in standard reaction mixture at varied pHs for 60 min. Reactions were terminated in a boiling water bath for 5 min and samples were stored frozen (-20 °C) until analysis. Following centrifugation (7,000 x g) to remove precipitated debris, capillary separation of MTX glutamated analogues was performed with a Spectra Phoresis 1000 instrument (Thermo Separation, San Jose, CA) with a 75 µm id x 50 cm silica capillary (Polymicro Technology, Phoenix, AZ). Separation of pteroyl(glutamate)_n derivatives is achieved with an electrolyte of 20 mM sodium borate with 15 mM sodium dodecylsulfate (pH 9.5) with +20 Kev at 25 °C. Samples were applied hydrodynamically for 1-2 s and absorbance monitored at 300 nm. Data were recorded with an IBM computer using CE-1000 software (Thermo Separation).

Protein determination: Protein concentrations of isolated membrane or supernatant fractions were

determined by incubating diluted aliquots with BCA reagent (Pierce Chemical Co., Rockford, IL) at 37 °C for 30 min. The spectrophotometric quantitation of protein was conducted by determining the absorbance at 562 nm against bovine serum albumin standard.

Statistical Analysis: Data were analyzed by using the Statgraphics version 4.0 program (Statistical graphics Corporation, Rockville, MD) and where summarized are expressed as mean \pm S.D. Student's unpaired t test was used to determine significance of differences.

Results:

Membrane fractions isolated from human prostate adenocarcinoma cells (LNCaP) were incubated using primarily MTXglu₃ as substrate. The time course of hydrolysis of the gamma-linked triglutamate derivative and the subsequent appearance of MTXglu₂, MTXglu₁, and MTX after 30, 60, 120, and 240 min of incubation are illustrated in Figure 82. The semipurified PSM antigen exhibits pteroyl poly gamma-glutamyl exopeptidase activity that progressively liberates all of the possible glutamates from MTXGlu₃ with accumulation of MTX.

The PSM antigen was immunoprecipitated in the presence of 7E11-C5 anti-prostate monoclonal antibody and the PSM antigen-antibody complex was adsorbed onto a Protein A Sepharose Gel column. Following twice washing of the sepharose beads with 2 mL volumes of buffer and re-solubilization of the antigen-antibody complex by adjusting the elution pH to 3.0, the supernatant fraction was assayed for hydrolase activity. Figure 55 shows the capillary electrophoretic separation of successively cleaved glutamyl moieties from MTXglu₃ after 0, 30, 60 and 240

min incubations. Results similar to these in Figure 82 were obtained using pteglu₁ with formation of folate (pteglu₁).

5 The optimum pH activity profiles of the immunoprecipitated PSM hydrolase from LNCaP cells and of the membrane fractions from PC-3 PSM-transfected and non-transfected (vector alone) cells are shown in Figure 57. The reaction was monitored as a function of
10 pH from 2 to 10 after an 1 h incubation with MTXglu₂. The extent of reaction was expressed as the concentration of MTXglu₂ formed per mg protein. Although all reaction products were detectable as illustrated in Figure 56, MTXglu₂ was the predominant
15 hydrolyzed species at incubation times ranging from 10 to 60 min. The pH profile of membrane fractions isolated from both LNCaP and PC-3 PSM-transfected cells are identical and exhibit two maxima of PSM hydrolase activity at pH 5 and 8 with no measurable activity
20 above pH 10.

To determine whether non-PSM expressing human adenocarcinoma cell lines (PC-3, TSU-Pr1, and Duke-145) exhibit folate hydrolase activity, isolated membrane
25 preparations from these cell lines were analyzed (Figure 83). The less differentiated, hormone refractory prostate cell lines (PC-3, TSU-Pr1, and Duke-145) exhibit no appreciable activity after 2 h incubations. These results are in agreement with
30 previous findings that demonstrate neither a presence of a mRNA for PSM nor antigen immunoreactivity with 7E11-C5 in these cells.

In further studies in which the cDNA for PSM was
35 transfected into non-PSM antigen expressing PC-3 cells, a close correlation between PSM antigen immunoreactivity and hydrolase activity was observed

with MTXglu₃ in membranes of LNCaP and PC-3 PSM-transfected cells (Figures 58 and 59). Immunohistochemical analyses of LNCaP (Figure 58) and PSM antigen expressing PC-3 (Figure 85B) cells revealed
5 distinct positive staining with 7E11-C5 anti-prostate monoclonal antibody. Figure 85C illustrates no immunoreactivity in PC-3 cells expressing the pREP7 hygromycin vector alone. In preparations of negative controls, all three cell lines were reacted with IgG2aK
10 rather than with 7E11-C5 antibody. No background staining resulted with the secondary antibody conjugated with alkaline phosphatase.

To compare PSM hydrolase activity with that of other
15 gamma-glutamyl hydrolases that either reside within the lysosome or are secreted as observed in several neoplastic cells, its reactivity in the presence of thiol-containing reducing agents, namely, reduced glutathione, homocysteine, and dithiothreitol (DTT),
20 and the thiol reagent, p-hydroxymercuribenzoate (PHMB), at concentrations ranging from 0.05 - 0.5 mM was observed. Of the reduced sulfhydryl derivatives, it was discovered that only DTT (≥ 0.2 mM) was slightly inhibitory ($86 \pm 3\%$ of control). Unlike gamma-linked
25 peptide hydrolase retained within the lysosome, PSM hydrolase activity was maintained in the presence of 0.5 mM PHMB.

The reactivity of PSM hydrolase against an α -glutamate dipeptide, N-acetyl- α -aspartylglutamate (NAAG), has
30 been investigated and that the PSM enzyme from either LNCaP or PSM transfected PC-3 cell membranes hydrolyses NAAG producing N-acetylaspartate and glutamate was observed. Furthermore, MTXglu₃, pteglu₃, and pABAglu₃
35 were potent inhibitors of the PSM-mediated NAAG hydrolysis.

Discussion:

Membrane-bound PSM antigen has pteroyl poly gamma-glutamyl carboxypeptidase (folate hydrolase) activity. Gamma-glutamyl hydrolase activity is also present in
5 lysosomes of cells and these enzymes may be responsible for regulating the length of exogenous and endogenous folyl polyglutamate chain lengths. A characteristic difference between these two hydrolases is that the PSM enzyme exhibits substantial activity at pH values 7.5
10 to 8.0 in addition to having an acidic pH 4.5 to 5 optimum. Moderate levels of hydrolase activity are present within LNCaP cytosolic compartment and may represent the short intracellular fragment of this class II enzyme. This reflects an interesting
15 situation in these cells where the majority of RNA codes for the membrane-bound enzyme that is localized extracellularly. The ratio of the mRNAs in these samples that code for the class II membrane and the cytosolic proteins is ten to one. In normal prostate
20 tissue, the mRNA coding for the membrane protein is only one-tenth that of the cytosolic form.

It is clear from this study that the prostate specific membrane antigen functions as a folate hydrolase and is
25 unique in that it has activity on both the gamma-linked as well as the alpha linked peptide bonds. This is interesting for a number of reasons. First in the normal prostate it was demonstrated that the majority of the mRNA encodes a protein, PSM', that is likely to
30 be cytosolic and would imply that it may be that in the prostate that folates could exist in the lesser glutamated species. If so then it means that the folate in the prostate can readily leak out and that the prostate may be subjected to "microenvironmental
35 folate deficiencies" This may be related to the high worldwide incidence of "microscopic prostate cancer" as folate deficiencies are associated with carcinogenesis

in a number of tissues.

Benign enlargement of the prostate and prostate cancer occur in older men. It also occurs that the uptake of folate decreases with aging. If folate uptake decreases with aging this may be due to decreased PSM folate hydrolase activity in the proximal intestine. To correct such a deficiency it might be possible to use PSM folate hydrolase in foods to release the folate before consumption or take it with foods as is done with lactase in lactose intolerant individuals. If the prostate in men is susceptible to folate depletion then nutritional supplementation may help reduce the development of the microscopic lesion, indeed in some cancers such as cancer of the colon, folate supplementation was found to reduce cancer formation.

Why would the prostate cells prefer to have the lesser glutamated forms of folate? It may be that methionine synthase which is an enzyme key to folate uptake and folate utilization for one carbon methyl transfer metabolism may utilize the nonglutamated folate preferentially. In addition to folate deficiency, choline and methionine deficiency is also associated with tumor development. If shown to modulate one carbon transfers, it might be useful to inhibit this enzyme as a means to inhibit cancer development and thus serve as a chemopreventative agent. Again modulation of PSM folate hydrolase may play a role in tumor prevention and modulation of tumor growth.

A feature that cell biologists use in transfecting DNA into cells often requires selection of the transfected gene and often multiple transfections are performed. These are done with drugs that are toxic to cells such as Hygromycin and use genes that code for Hygromycin resistance which are bacterial. It may be that PSM

could be used as a selectable marker by growing the transfected cells in folate free media and including polyglutamated folate which would be able to rescue cells from folate deficiency if they expressed PSM.

5

PSM folate hydrolase activity can possibly be used as a prodrug converting enzyme. In the normal prostate PSM is intracellular. In the transformed cell the majority of the protein and its attendant enzymatic activity is extracellular in location. It may be that as the enzymes associated with cell growth require the polyglutamated forms the cancer finds a way to remove PSM folate hydrolase from the interior by alternative splicing to an extracellular enzyme. PSM is a membrane protein and is found to predominate in cancer, but PSM' is likely a cytosolic protein which predominates in the normal condition.

20 This implies that development of a prodrug that requires metabolism before it can be taken up by the tumor cell could be activated by the PSM folate hydrolase which is predominate in the cancer.

25 Methotrexate triglutamate was one of the agents used to identify the enzymatic activity of PSM antigen. Methotrexate triglutamate would not be able to use the transport protein to be taken into tumor cells, because there are specific structural requirements for folate, or methotrexate transport. If one removes the gamma-linked glutamates then methotrexate can be taken into cells and can exerts its antifolate, antitumor growth action.

35 Therefore methotrexategammatriglutamate was used to examine the action of this compound on the in vitro growth of PC-3 cells transfected with a plasmid with a

selectable marker versus a plasmid with a selectable
marker that expresses PSM antigen as well. the PC-3
cells that were transfected with PSM were inhibited 85%
in growth by day four by 10uM methotrexate
5 triglutamate, while the PC-3 plasmid only transfectants
did not exhibit any significant inhibition of growth.

PSM's folate hydrolase activity hydrolyses down to the
last glutamate which is in alpha linked position but
10 does not remove it. Because it does not remove the last
glutamate, PSM antigen's folate hydrolase activity
better serves the prodrug activation requirements of
such a prodrug. Also because it is a human enzyme it
is less likely than the carboxypeptidase G2 will cause
15 an immune response because PSM antigen is normally
present in the body.

In addition PSM could also be used as part of a prodrug
strategy that utilized gene transfer and a tissue or
20 tumor specific promoter, say such that it would be
linked to CEA promoter and PSM expressed in colon
tumors and the patients subsequently given the prodrug
such as methotrexate triglutamate. The same is also
true for the protein itself, either the whole protein
25 or the components of the active site or a modified
version that would have increased prodrug activating
activity could be linked to a delivery vehicle such as
an antibody or other specific targeting ligand,
delivered to the tumor for localization and subsequent
30 activation.

Methotrexate as a prodrug may be enhanced in
specificity by using alpha linked glutamates rather
than gamma linked glutamates because the ubiquitous
35 lysosomal hydrolase enzyme is specific for the gamma
linked bond. A pro-drug with all alpha linked
glutamates would not be a substrate, but would be a

substrate for the PSM folate hydrolase.

5 In addition to methotrexate a number of potential enzyme substrates can be employed as cytotoxic prodrugs. The synthesis of potential prodrugs, PALAgly, and a number of other potential agents are described.

10 Alpha-linked methotrexate material is synthesized by the following Merifield solid phase scheme (see Figure 88). The scheme is based on a modification of the standard Merifield solid peptide synthesis that was applied to the synthesis of methotrexate γ polyglutamates. In brief the N-Fmoc-4-terbutylglutamate
15 is first connected to the resin under standard coupling conditions using diisopropylazodicarboxylate as a coupling reagent. The Fmoc protecting group is then removed with piperidine, and this cycle would be reiterated for as many times as glutamates would be
20 needed to obtain the desired analog. For example say the pentaglutamate on solid support is the intermediate required for the preparation of methotrexate-alpha-tetraglutamate. It is deprotected at the terminal nitrogen by treatment with piperidine, then coupled
25 with pteronic acid analogue under the same conditions used above. The terbutyl and the resin are all removed in one step with 95% trifluoroacetic acid (TFA) to provide the desired material. This process is applied to every analog. The gamma linked material is provided
30 in a similar manner for use comparative studies with the alpha-linked material (see figure 89). Because of the carboxypeptidase activity a number of combination of alpha and gamma linked acidic amino acid can be optimized for their utilization of the enzyme and for
35 in vivo activity. In addition to the folate like antagonists, a number of amino acid analogs were found in the past to have antitumor activity but lacked in

vivo specificity. These agents are targetable by attaching a glutamate to the carboxy terminus of the amino acid as described and shown in the figures.

5 PALA-Glutamate 3 and analog 5, was synthesized in a similar manner with the addition to the introduction of a protected phosphonoacetate moiety instead of a simple acetate. It is compatible with the function of diethylphosphonoacetic acid which allows the removal of
10 the ethyl groups under relatively mild conditions.

Commercially available diethylphosphonoacetic acid was treated with perfluorophenyl acetate in pyridine at 0 deg.C to room temperature for an hour to afford the
15 corresponding pentafluorophenyl ester in nearly quantitative yield after short path column chromatography. This was then reacted with gamma-benzylaspartate and HOAT in tetrahydrofuran for half an hour at reflux temperature to give protected PALA 7 (N-phosphonoacetylaspargate) in 90% yield after flash
20 column chromatography. The free acid was then activated as its pentafluorophenyl ester 8, then it was reacted with delta-benzyl-L-glutamate and HOAT in a mixture of THF-DMF (9:1, v/v) for 12 hours at reflux to
25 give fully protected PALA-Glutamate 9 in 66% yield after column chromatography. Sequential removal of the ethyl groups followed by the debenzylation was accomplished for a one step deprotection of both the benzyl and ethyl groups. Hence protected PALA-
30 Glutamate was heated up to reflux in neat trimethylsilylchloride for an overnight period. The resulting bistrimethylsilylphosphonate ester 10 was submitted without purification to hydrogenolysis (H_2 , 30 psi, 10% Pd/C, ethylacetate). The desired material 3
35 was isolated after purification by reverse phase column chromatography and ion exchange resin.

Analog 4 and 5 were synthesized by preparation of phosphonoglutamate 14 from the alpha-carboxyl-protected glutamate.

5 Commercially available alpha-benzyl-N-Boc-L-glutamate
11 was treated at refluxing THF with neat
boranedimethylsulfide complex to afford the
corresponding alcohol in 90% yield. This was
transformed into bromide 12 by the usual procedure
10 ($\text{PPh}_3, \text{CBr}_4$).

The Michaelis-Arbuzov reaction using triethylphosphite
to give the corresponding diethylphosphonate 13 which
would be deprotected at the nitrogen with
15 trifluoroacetic acid to give free amine 14. The latter
would be condensed separately with either
pentafluorophenylesters 6 or 8 to give 16 and 15
respectively, under conditions similar to those
described for 3. 15 and 16 would be deprotected in the
20 same manner as for 3 to yield desired analogs 4 and 5.

An inhibitor of the metabolism of purines and
pyrimidine like DON (6-diazo-5-oxo-norleucine) or its
aspartate-like 17, and glutamate-like 18 analogs would
25 be added to the series of substrates.

Analog 20 is transformed into compound 17 by treatment
with oxalyl chloride followed by diazomethane and
deprotection under known conditions to afford the
30 desired analogs. In addition, azotomycin is active only
after in vivo conversion to DON which will be released
after action of PSM on analogs 17, 18, and 19.

Representative compounds, 21 and 22, were designed
35 based on some of the specific effects and properties of
PSM, and the unique features of some newly discovered
cytotoxic molecules with now known mode of action. The

latter, referred to commonly as enediynes, like
dynemycin A 23 and or its active analogs. The recent
isolation of new natural products like Dynemycin A 23,
has generated a tremendous and rapidly growing interest
5 in the medical and chemical sciences. They have
displayed cytotoxicities to many cancer cell lines at
the sub-nanomolar level. One problem is they are very
toxic, unstable, and non-selective. Although they have
been demonstrated, in vitro, to exert their activity
10 through DNA damage by a radical mechanism as described
below, their high level of toxicity might imply that
they should be able to equally damage anything in their
path, from proteins to enzymes.

15 These molecules possess unusual structural features
that provide them with exceptional reactivities.
Dynemycin A 23 is relatively stable until the
anthraquinone moiety is bio-reduced into
hydroanthraquinone 24. This triggers a chain of events
20 by which a diradical species 25 is generated as a
result of a Bergman cycloaromatization^f. Diradical
species 25 is the ultimate damaging edge of dynemycin
A. It subtracts 2(two) protons from any neighboring
molecule or molecules(ie. DNA) producing radicals
25 therein. These radicals in turn combine with molecular
oxygen to give hydroperoxide intermediates that, in the
case of DNA, lead to single and double strand incision,
and consequent cell death. Another interesting feature
was provided by the extensive work of many organic
30 chemists who not only achieved the total synthesis of
(+)-dynemycin A 23 and other enediynes. but also
designed and efficiently prepared simpler yet as active
analogs like 26.

35 Enediyne 26 is also triggerable and acts by virtue of
the same mechanism as for 23. This aspect is very
relevant to the present proposed study in that 27 (a

very close analog of 26) is connected to NAAG such that the NAAG-27 molecule, 21, would be inert anywhere in the body (blood, organs, normal prostate cells) except in the vicinity of prostate cancer, and metastatic cells. In this connection NAAG plays a multiple role:

- Solubilization and transport: analogs of 26-type are hydrophobic and insoluble in aqueous media, but with a water soluble dipeptide that is indigenous to the body, substrate 21 should follow the ways by which NAAG is transported and stored in the body.

- Recognition, guidance, and selectivity: Homologs of PSM are located in the small intestines and in the brain.

In the latter, a compound like 27 when attached to a multiply charged dipeptide like NAAG, has no chance of crossing the blood brain barrier. In the former case, PSM homolog concentration in the small intestines is in the brush border and not likely to be exposed to prodrugs in the serum. In addition, one could enhance the selectivity of delivery of the prodrug by local injection in the prostate.

26 and its analogs are established active molecules that portray the activity of dynemycin A. Their syntheses are described in the literature. The total synthesis of optically active 27 has been described⁶. The synthetic scheme that for the preparation of 28 is almost the same as that of 27. However, they differ only at the position of the methoxy group which is meta to the nitrogen in the case of 28. This requires an intermediate of type 29 prepared by modification of the Myers' method.

Since NAAG is optically pure, its combination with

racemic material sometimes complicates purification of intermediates. In addition, to be able to modify the components of this system one at a time, optically pure intermediates of the type 21 and 22 are prepared. 27
5 was prepared in 17 steps starting from commercially available material. Another interesting feature of 27 is demonstrated in a very close analog 26, it possesses two(2) triggers as shown by the arrows.

10 The oxygen and the nitrogen can both engender the Bergman cycloaromatization and hence the desired damage. The simple protection deprotection manipulation of either functionality should permit the selective positioning of NAAG at the nitrogen or at the
15 oxygen centers. PSM should recognize the NAAG portion of 21 or 22, then it would remove the glutamic acid moiety. This leaves 27 attached to N-acetylaspartate.

Intramolecular assisted hydrolysis of systems like N-
20 acetylaspartyle is well documented in the literature. The aminoacid portion should facilitate the hydrolysis of such a linkage. In the event this would not work when NAAG is placed on the nitrogen, an alternative would be to attach NAAG to the oxygen giving rise to
25 phenolic ester 22 which is per se labile and removable under milder conditions. PSM specific substrates can be designed that could activate pro-drugs at the site of prostatic tumor cells to kill those cells.

EXAMPLE 13:

GENOMIC ORGANIZATION OF PSM EXON/INTRON JUNCTION
5 SEQUENCES

RNA is synthesized and then processed by having
variable numbers of variable sized fragments cut out
and remain in the nucleus (introns) and the remaining
10 fragments (exons) joined together and transported out
of the nucleus (mRNA) for use in translation into
protein in the cytoplasm. This mRNA is what make the
unique protein products of the cell, proteins of
specialized cells are often made in a great abundance
15 as are their respective coding mRNA's. These tissue
specific mRNA's can be reverse transcribed (RT) into DNA
by reverse transcriptase and amplified for detection by
polymerase chain reaction (PCR) technology and thus the
technique is called RT-PCR. If DNA is a contaminant of
20 the MRNA fraction it would contain the message even
though it was not being transcribed.

Knowledge of the intron exon junctions allows for the
selection of primer pairs that cross an intron junction
25 and thus allow the determination of DNA contamination
of the RNA preparation, if present. If the intron
junction were large it would be unlikely to be
amplified with primers, while if the intron junction
were small it would still produce a fragment that would
30 be much larger than the predicted fragment size which
is based on the cDNA sequence. Thus knowledge of the
intron/exon junctions provides a control to determine
if the RT-PCR product is contaminated with DNA.
Another form of DNA that could also be amplified
35 undesirably if present as a contaminant are pseudo
genes, which are intronless forms of the mRNA that
reside as DNA but are not expressed as RNA. Thus,

optimized primers for detection of PSM mRNA in samples would preferably contain sequences hybridizing across the intro/exon junctions which are as follows:

```
5
      EXON 1      Intron 1
1F. strand
CGGCTTCCTCTTCGG
cggcttcctcttcgg taggggggcgcctcgcgag...tatttttca
10
1R. strand                      ...ataaaaagtCACCAA

      Exon 2      Intron 2
15 2F. strand
ACATCAAGAAGTTCT
acatcaagaagttct caagtaagtcatactcgaag...

2R. strand                      ...caagtggcATATATTAAAATG
20

      Exon 3      Intron 3
3F. strand
GAAGATGGAAATGAG
25 gaagatggaaatgag gtaaaatataaataaataaataa...

3R.                      ...TAAAAGTTGTGTAGT

      Exon 4      Intron 4
30 4F. strand
AAGGAATGCCAGAGG
aaggaatgccagagg taaaaacacagtgcaacaaa...

4R. strand                      ...agagttgCCGCTAGATCACA
35
```

Exon 5 Intron 5

5F. strand
CAGAGGAAATAAGGT
cagaggaaataaggt aggtaaaaattatctctttttt...

5 5R. strand ...gtgttttctATTTTACGGGT

10 Exon 6 Intron 6

6F. strand
GTTACCCAGCAAATG
gttaccagcaatg gtgaatgatcaatccttgaat...

15 6R. strand ...aaaaaaagtTTATACGAATA

Exon 7 Intron 7

7F. strand
20 ACAGAAGCTCCTAGA
acagaagctcctaga gtaagtttgtaagaaaccargg...

7R. strand ...aaacacaggttatcTTTTACCCA

25 Exon 8 Intron 8

8F. strand
AAACTTTTCTACACA
aaacttttctacaca gttaagagactatataaatttta...

30 8R. strand aacgtaatcaTTTTCAGTTCTAC

Exon 9 Intron 9

9F. strand
AGCAGTGAACCAG
35 agcagtggaaccag gtaaaggaatcgtttgctagca...

9R. strand ...aaagaTGTCTATACAGTAA

Exon 10 Intron 10
10F. Strand
CTGAAAAAGGAAGG
ctgaaaaaggaagg taatacaaacaaatagcaagaa...
5

Exon 11 Intron 11
11F. Strand
TGAGTGGGCAGAGG
10 agagg ttagttggttaatttgctataatata...

Exon 12 Intron 12
12F. strand
15 ATCTATAGAAGG
gtagtttct gaaaaataagaaaagaatagat...

Exon 13 Intron 13
20 13F. strand
CTAACAAAAGAG
agggccttttcagct acacaaattaaaagaaaaaaag...

25 Exon 14 Intron 14
14F. strand
GTGGCATGCCCAGG
gtggcatgcccagg taaataaatgaatgaagtttcca...

30 Exon 15 Intron 15
15F. strand
CTAAAAATTGGC
aatttgtttgtttcc tacagaaaaacaacaaaaca...
35

Exon 16 Intron 16

16F. strand
CAGTGTATCATTTG
cagtgtatcatttg gtatggtacccttcctttttcaaatt...

5 16R. strand ...aaagtcTAAGTGAAAA

Exon 17 Intron 17

10 17F. strand
TTTGACAAAAGCAA
tttgacaaaagcaa gtatggtctacatatatgtgcatat...

15 17R. strand ...aaagagtcGGGTTATCAT

Exon 18 Intron 18

18F. strand
GGCCTTTTTATAGG
20 ggcctttttatagg taaganaagaaaatatgactcct...

18R. strand ...aatagttgGTACAGTAGATA

25 Exon 19 Intron 19

19F. strand
GAATATTATATATA
gaatattatatata gttatgtgagtggtttatatatgtgtgt...

30

Notes: F: Forward strand
R: Reverse strand

REFERENCES:

1. Abdel-Nabi, H., Wright, G.L., Gulfo, J.V.,
5 Petrylak, D.P., Neal, C.E., Texter, J.E.,
Begun, F.P., Tyson, I., Heal, A., Mitchell,
E., Purnell, G., and Harwood, S.J.
Monoclonal antibodies and
10 radioimmunoconjugates in the diagnosis and
treatment of prostate cancer. Semin. Urol.,
10: 45-54, 1992.
2. Antonie, P. Springer, C.J., Bagshawe, F.,
Searle, F., Melton, R.G., Rogers, G.T.,
15 Burke, P.J., Sherwood, R.F. Disposition of
the prodrug 4-bis(2chloroethyl) amino)
benzoyl-L-glutamic acid and its active
parent drug in mice. Br.J.Cancer 62:909-
914, 1990.
- 20 3. Aviv, H., and Leder, P. Purification of
biologically active globin messenger RNA by
chromatography on oligo-thymidylic acid
cellulose. Proc. Natl. Acad. Sci. USA, 69:
25 1408-1412, 1972.
4. Axelrod, H.R., Gilman, S.C., D'Aleo, C.J.,
Petrylak, D., Reuter, V., Gulfo, J.V., Saad,
A., Cordon-Cardo, C., and Scher, H.I.
30 Preclinical results and human
immunohistochemical studies with ⁹⁰Y-CYT-356;
a new prostatic cancer therapeutic agent.
AUA Proceedings, Abstract 596, 1992.
- 35 5. Boring, C.C., Squires, T.S., Tong, T., and
Montgomery, S. Cancer Statistics, 1994.
CA., 44: 7-26, 1994.

6. Chiarodo, A. National Cancer Institute roundtable on prostate cancer; future research directions. Cancer Res., 51: 2498-2505, 1991.
7. Chiaroda, A. (1991) National roundtable of prostate cancer: research directions. Cancer Res. 51: 2498-2505.
8. Coffey, D.S. Prostate Cancer - An overview of an increasing dilemma. Cancer Supplement, 71,3: 880-886, 1993.
9. Connor, J. Bannerji, R., Saito, S., Heston, W.D.W., Fair, W.R., Gilboa, E. Regression of bladder tumors in mice treated with interleukin 2 gene-modified tumor cells. J.Exp.Med. 177:1127-1134, 1993. (appendix)
10. Deguchi, T., Doi, T., Ehara, H., Ito, S., Takahashi, Y., Nishino, Y., Fujihira, S., Kawamura, T., Komeda, H., Horie, M., Kaji, H., Shimokawa, K., Tanaka, T., and Kawada, Y. Detection of micrometastatic prostate cancer cells in lymph nodes by reverse-transcriptase polymerase chain reaction. Cancer Res. 53:5350-4, 1993.
- 11.
12. Eisenburg, D., Schwarz, E., Komaromy, M. and Wall, R. Analysis of membrane and surface protein sequences with the hydrophobic moment plot, J. Mol. Biol. 179:125-142, 1984.
13. Feinberg, A.P., and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific

activity. Anal. Biochem., 132:6-13, 1983.

14. Feng, Q., et al., (1991) Purification and
5 biochemical characterization of the 7E11-C5
prostate carcinoma associated antigen. Proc.
Amer. Assoc. Cancer Res. 32:239.
15. Fey, M.F., Kulozik, A.E., and Hansen-Hagge,
10 T.E.: The polymerase chain reactipn: A new
tool for the detection of minimal residual
disease in hematological malignacies. Eur.
J. Cancer, 27: 89-94, 1991.
16. Gussow, D., Rein, R., Ginjaar, I.,
15 Hochstenbach, F., Seemann, G., Kottman, A.,
Ploegh, H.L. The human β -2-Microglobulin
gene. Primary structure and definition of
the transcriptional unit. J. of Immunol.
139:3132-3138, 1987.
- 20 17. Glisin, V., Crkvenjakov, R., and Byus, C.
Ribonucleic acid isolated by cesium chloride
centrifugation. Biochemistry, 13: 2633-
2637, 1974.
- 25 18. Ghossein, R., Scher, H., Gerald, W.,
Hoffman, A., Kelley, W., Curely, T.,
Libertz, C., and Rosai, J. Detection of
30 cirulating tumor cells in peripheral blood
of patients with advanced prostatic
carcinoma. Proc. Amer. Soc. of Clin.
Oncol., 13:237, 1994.
- 35 19. Hanahan, D.: Studies on transformation of
Escherichia coli with plasmids. J. Mol.
Biol., 166:557-580, 1983.

20. Harlow, E., and Lane, D. Antibodies: A Laboratory Manual. New York: Cold Spring Harbor Laboratory, p. 449, 1988.
- 5 21. Henttu, P., et al., (1989) cDNA coding for the entire human prostate specific antigen show high homologies to the human tissue kallikrein genes. Bioch. Biophys. Res. Comm. 160:903-908.
- 10 22. Horoszewicz, J.S., Kawinski, E., and Murphy, G.P. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. Isaeli, R.S., Powell, C.T., Fair, W.R., and Heston, W.D.W.: Molecular cloning of a complementary DNA encoding a prostate-specific membran antigen. Cancer Res., 53: 227-230, 1993.
- 15 23. Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Chu, T.M., Mirand, E.A., and Murphy, G.P.: LNCaP model of human prostatic carcinoma. Cancer Res., 43: 1809-1818, 1983.
- 20 25. Anticancer Res., 7: 927-936, 1987.
- 25 24. Horoszewicz, J.S., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Chu, T.M., Mirand, E.A., and Murphy, G.P. LNCaP model of human prostatic carcinoma. Cancer Res., 43: 1809-1818, 1983.
- 30 25. Horoszewicz, J.S., Kawinski, E., and Murphy, G.P. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients.
- 35

Anticancer Res., 7:927-936,1987.

26. Horoszewicz, J.S., Leong, S.S., Kawinski,
E., Karr, J.P., Rosenthal, H., Chu, T.M.,
5 Mirand, E.A. and Murphy, G.P. LNCaP model
of human prostatic Carcinoma. Cancer Res.,
43:1809-1818,1983. 8.
27. Horoszewicz, J.S., et al. (1987) Monoclonal
10 antibodies to a new antigenic marker in
epithelial prostatic cells and serum of
prostatic cancer patients. Anticancer Res.
7:927-936.
28. Horoszewicz, J.S., et al. (1983) LNCaP model
15 of human prostatic carcinoma. Cancer Res.,
43:1809-1818.
29. Hsu, S.M., Raine, L., and Fanger, H. Review
20 of present methods of immunohistochemical
detection. Am. J. Clin. Path. 75: 734-738,
1981.
30. Israeli, R.S., Powell, C.T., Fair, W.R., and
25 Heston, W.D.W. Molecular cloning of a
complementary DNA encoding a prostate-
specific membrane antigen. Cancer Res., 53:
227-230, 1993.
31. Israeli, R.S., Miller Jr., W.H., Su, S.L.,
30 Powell, C.T., Fair, W.R., Samadi, D.S.,
Huryk, R.F., DelBlasio, A., Edwards, E.T,
and Heston, W.D.W. Sensitive Nested Reverse
Transcription Polymerase Chain Reaction
35 Detection of Circulating Prostatic Tumor
Cells: Comparision of Prostate-specific
Membrane Antigen and Prostate-specific

Antigen-based Assays. Cancer Res., 54:
6325-6329, 1994.

- 5 32. Israeli, R.S., Powell, C.T., Fair, W.R.
and Heston, W.D.W. Molecular cloning of a
complementary DNA encoding a prostate-
specific membrane antigen. Cancer
Res., 53:227-230, 1993.
- 10 33. Israeli, R.S., Powell, C.T., Corr, J.G.,
Fair, W.R. and Heston, W.D.W. Expression of
the prostate-specific membrane antigen.
Cancer Res., 54:1807-1811, 1994.
- 15 34. Israeli, R.S., Miller, W.H., Jr., Su, S.L.,
Samadi, D.S., Powell, C.T., Heston, W.D.W.,
Wise, G.J., and Fair, W.R. Sensitive
detection of prostatic hematogenous
20 micrometastases using prostate-specific
antigen (PSA) and prostate-specific membran
antigen (PSM) derived primers in the
polymerase chain reaction. J. Urol.
151:373A, 1994.
- 25 35. Israeli, R.S., Powel, C.T., Corr, J.G.,
Fair, W.R., and Heston, W.D.W.: Expression
of the prostate-specific membrane antigen.
Cancer Res., 54:1807-1811, 1994.
- 30 36. Israeli, R.S., Miller, W.H., Jr., Su, S.L,
Samadi, D.S., Powell, C.T. Heston, W.D.W.,
Wise, G.J., and Fair, W.S. Sensitive
detection of prostatic hematogenous
35 micrometastases using PSA and PSM-derived
primers in the polymerase chain reaction.
In press - J. Urology.

37. Kaign, M.E., Narayan, K.S., Ohnuki, Y., and
Lechner, J.F. Establishment and
characterization of a human prostatic
carcinoma cell line (PC-3). Invest. Urol.,
5 17: 16-23, 1979.
38. Katz, A.E., Olsson, C.A., Raffo, A.J., Cama,
C., Perlman, H., Seaman, E., O'Toole, K.M.,
McMahon, D., Benson, M., and Buttyan, R.,
10 Molecular staging of prostate cancer with
the use of an enhanced reverse
transcriptase-PCR assay. Urology 43:765-
775, 1994.
39. Liotta, L.A. (1986) Tumor invasion and
metastases: role of the extracellular
matrix. Cancer Res. 46:1-7.
15
40. Liotta, L.A., Kleinerman, J., and Saidel,
G.M.: Quantitative relationships of
intravascular tumor cells, tumors vessels,
and pulmonary metastases following tumore
implantation. Cancer Res., 34:997-1003,
1974.
20
41. Lopes, D., et al. (1990) Immunohistochemical
and pharmacokinetic characterization of the
site-specific immunoconjugate CYT-356,
derived from anti-prostate monoclonal
antibody 7E11-C5. Cancer Res., 50:6423-
6429.
25 30
42. Lopes, A.D., Davis, W.L., Rosenstrauss, M.J.,
Uveges, A.J., and Gilman, S.C.
Immunohistochemical and pharmacokinetic
characterization of the site-specific
immunoconjugate CYT-356 derived from
35

antiprostata monoclonal antibody 7E11-C5.
Cancer Res., 50: 6423-6429, 1990.

- 5 43. Lundwall, A., and Lilja, H: Molecular
cloning of a human prostate specific antigen
cDNA. FEBS Letters, 214: 317, 1987. 7.
- 10 44. Melton, D.A., Krieg, P.A., Rebagliati, M.R.,
Maniatis, T.A., Zinn, K., and Careen, M.R.
Efficient in-vitro synthesis of biologically
active RNA and RNA hybridization probes from
plasmids containing a bacteriophage SP6
promoter. Nucl. Acids. Res. 12: 7035-7056,
1984.
- 15 45. Miller, W.H., Jr., Levine, K., DeBlasio, A.,
Frankel, S.R., Dmitrovsky, E., and Warrell,
R.P., Jr. Detection of minimal residual
disease in Acute Promyelocytic Leukemia by
20 a reverse transcription polymerase chain
reaction assay for the PML/RAR- α fusion mRNA.
Blood, 82: 1689-1694, 1993. Moreno, J.G.,
Croce, C.M., Fischer, R., Monne, M., Vihko,
P., Mulholland, S.G., and Gomella, L.G.,
25 Detection of hematogenous micrometastasis in
patients with prostate cancer. Cancer Res.,
52:6110-6112, 1992.
- 30 46. Murphy, G.P. Report on the American Urologic
Association/American Cancer Society
Scientific Seminar on the Detection and
treatment of Early-Stage Prostate Cancer. CA
Cancer J. Clin. 44:91-95,1994.
- 35 47. Nguyen, L., et al., (1990) Prostatic acid
phosphatase in the serum of cancer patients
with prostatic cancer is a specific

phosphotyrosine acid phosphatase. Clin.
Chem. 35:1450-1455.

- 5 48. Oberneder, R., Riesenberger, R., Kriegmair,
M., Bitzer, U., Klammert, R., Schneede, P.,
Hofstetter, A., Riethmuller, G., and Pantel,
K. Immunocytochemical detection and
phenotypic characterization of
10 micrometastatic tumour cells in bone marrow
of patients with prostate cancer. Urol.
Res. 22:3-8, 1994.
- 15 49. Rao, M.J.K. and Argos, P. A conformational
preference parameter to predict helices in
integral membrane proteins. Biochim.
Biophys. Acta, 869:197-214, 1986.
- 20 50. Roemer, K., Friedmann, T. Concepts and
strategies for human gene therapy. FEBS.
223:212-225.
- 25 51. Sanger, F., Nicklen, S., and Coulson, A.R.:
DNA sequencing with chain-terminating
inhibitors. Proc. Natl. Acad. Sci. USA,
74:5463-5467, 1977.
- 30 52. Soule, H.D., Vazquez, J., Long, A., Albert,
S., and Brennan, M.: A human cell line from
a pleural effusion derived from a breast
carcinoma. J. Natl. Can. Inst., 51: 1409-
1416, 1973.
- 35 53. Stone, K.R., Mickey, D.D., Wunderli, H.,
Mickey, G.H., and Paulson, D.F. Isolation of
a human prostate carcinoma cell line (DU-
145). Int. J. Cancer, 21: 274-281, 1978.

54. Troyer, J.K. and Wright Jr., G.L. Biochemical characterization and mapping of 7E-11 C-5.3. Epitope of the prostate specific membrane antigen (PSMA). American Association for Cancer Research Special Conference: Basic and Clinical Aspect of Prostate Cancer. Abstract C-38, 1994.
55. Troyer, J.K., Qi, F., Beckett, M.L., Morningstar, M.M., and Wright, G.L. Molecular characterization of the 7E11-C5 prostate tumor-associated antigen. AUA Proceedings. Abstract 482, 1993.
56. Vessella, R., Stray, J., Arman, E., Ellis, W., and Lange, P. Reverse transcription polymerase chain reaction (RT-PCR) detects metastatic prostate cancer cells in lymph nodes, blood and potentially bone marrow using PSA-mRNA as template, J. Urol. 151:412A, 1994.
57. Vile R., Hart, I.R. In vitro and in vivo targeting of gene expression to melanoma cells. Cancer Res. 53:962-967, 1993.
58. Vile, R.G., Hart, I.R. Use of tissue specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoral injection of DNA. Cancer Res. 53:3860-3864, 1993.
59. Warner, J.A., Heston, W.D.W. Future developments of nonhormonal systemic therapy for prostatic carcinoma. Urologic Clinics of North America 18:25-33, 1991.

60. Warner, J.A., et al., (1991) Future
developments of non-hormonal systemic
therapy for prostatic carcinoma. Urologic
Clin. North Amer. 18:25-33.
61. Wright, Jr., et al., (1990) Characterization
of a new carcinoma associated marker:7E11-
C5. Antibod. Immunoconj.
Radiopharm.3:(abst#193).
62. Wu, A., Ben-Ezra, J., and Colombero, A.:
Detection of micrometastasis in breast
cancer by the polymerase chain reaction.
Lab. Invest., 62: 109A, 1990.
63. Wood, D.P., Jr., Banks, E.R., Humphries, S.,
McRoberts, J.W., and Rangenkar, V.M.
Identification of micrometastases in
patients with prostate cancer. J. Urol.
151:303A, 1994.
64. Wright, G.L., Jr., Haley, C., Beckett, M.L.,
and Schellhammer, P.F. Expression of the
prostate biomarker 7E11-C5 in primary and
metastatic prostate carcinoma. Proc. Amer.
Ass. for Can. Res. 35:233, 1994.
65. Yong, CY-F., et al., (1991) Hormonal
regulation of prostate-specific antigen
messenger RNA in human prostatic
adenocarcinoma cell line LNCaP. Cancer Res.
51:3748-3752.

What is claimed is:

1. An isolated nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen.
2. An isolated DNA of claim 1.
3. An isolated cDNA of claim 2.
4. An isolated RNA of claim 1.
5. An isolated DNA of claim 2 operatively linked to a promoter of RNA transcription.
6. A vector which comprises the nucleic acid of claim 1.
7. A host vector system for the production of a polypeptide having the biological activity of the alternatively spliced prostate-specific membrane antigen which comprises the vector of claim 6 and a suitable host.
8. A host vector system of claim 7, wherein the suitable host is a bacterial cell, insect cell, or mammalian cell..
9. An isolated nucleic acid comprising a promoter sequence normally associated with the transcription of a gene encoding a human prostate-specific membrane antigen.
10. An isolated polypeptide having the biological activity of an alternatively spliced prostate-specific membrane antigen.

11. An antibody which specifically binds to the polypeptide of claim 10.
- 5 12. The antibody of claim 11, wherein the antibody is monoclonal antibody.
13. The antibody of claim 11, wherein the antibody is polyclonal antibody.
- 10 14. The antibody of claim 11, wherein the antibody is labelled with a detectable marker.
- 15 15. The labelled antibody of claim 14, wherein the marker is radioactive, or colorimetric, luminescent, or fluorescent marker.
- 20 16. A method of detecting in a sample the presence of a nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen which comprises: a) obtaining a suitable sample; b) extracting RNA from the sample; c) contacting the RNA with reverse transcriptase under suitable conditions to obtain a cDNA; d) contacting the cDNA under hybridizing conditions with two
25 oligonucleotide primers,
 - 30 i) the first primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 3' of nucleotide 114 of such DNA sequence, with the proviso that the 3' end of the primer does not hybridize to any sequence located 5' of nucleotide 114, and
35 ii) the second primer being capable of specifically hybridizing to a sequence

5 within a DNA sequence encoding prostate
 specific membrane antigen located
 immediately 5' of nucleotide 381 of such DNA
 sequence, with the proviso that the 5' end
 of the primer does not hybridize to any
 sequence located 3' of nucleotide 381;

10 d) amplifying any cDNA to which the primers
 hybridize to so as to obtain amplification
 product; e) determining the size of the
 amplification product; f) comparing the size of
 the amplification product to the size of the
 amplification product known to be obtained using
15 the same primers with a non alternatively spliced
 human prostate specific membrane antigen, wherein
 a smaller amplification product is indicative of
 the presence of the alternatively spliced
 prostate specific membrane antigen, so as to
20 thereby detect the presence of the alternatively
 spliced human prostate-specific membrane antigen
 in the sample.

17. A method of detecting a prostate tumor cell in a
25 subject which comprises: which comprises: a)
 obtaining a suitable sample; b) extracting RNA
 from the sample; c) contacting the RNA with
 reverse transcriptase under suitable conditions
 to obtain a cDNA; d) contacting the cDNA under
 hybridizing conditions with two oligonucleotide
30 primers,

 i) the first primer being capable of
 specifically hybridizing to a sequence
 within a DNA sequence encoding prostate
35 specific membrane antigen located
 immediately 3' of nucleotide 114 of such
 DNA sequence, with the proviso that the 3'

end of the primer does not hybridize to any sequence located 5' of nucleotide 114, and

5 ii) the second primer being capable of
specifically hybridizing to a sequence
within a DNA sequence encoding prostate
specific membrane antigen located
immediately 5' of nucleotide 381 of such DNA
sequence, with the proviso that the 5' end
10 of the primer does not hybridize to any
sequence located 3' of nucleotide 381;

d) amplifying any cDNA to which the primers
hybridize to so as to obtain amplification
15 product; e) determining the amount of the
amplification product; f) comparing the amount
of the amplification product to the amount of
the amplification product known to be obtained
using the same primers with a non alternatively
20 spliced human prostate specific membrane antigen,
wherein a greater amount of the prostate specific
membrane antigen is indicative of a prostate
tumor cell in the subject, so as to thereby
detect prostate tumor cell in the subject

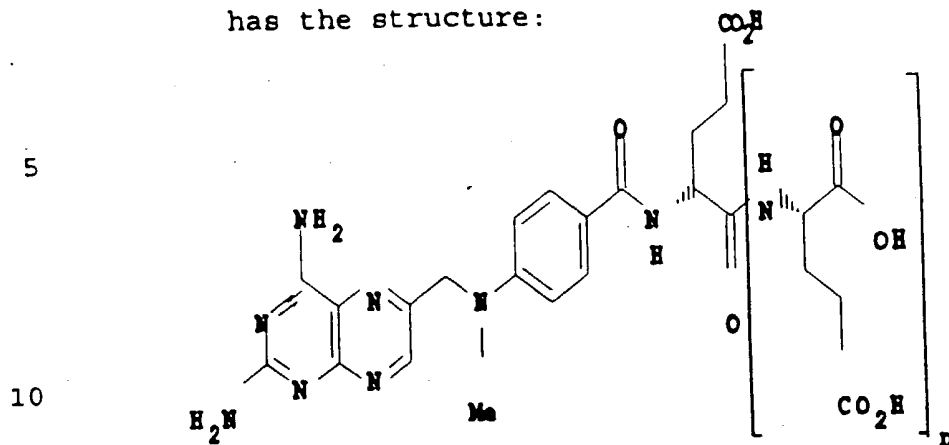
25

18. A compound comprising a conjugate of a cytotoxic
agent and one or more amino acid residues,
wherein each amino acid residue is glutamate or
aspartate.

30

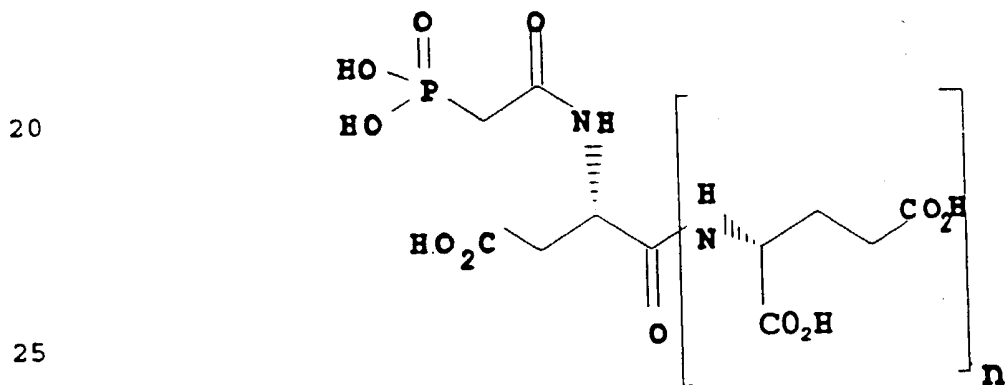
35

19. The compound of claim 18, wherein the compound has the structure:



wherein n is an integer from 1-10 inclusive.

- 15 20. The compound of claim 18, wherein the compound has the structure:

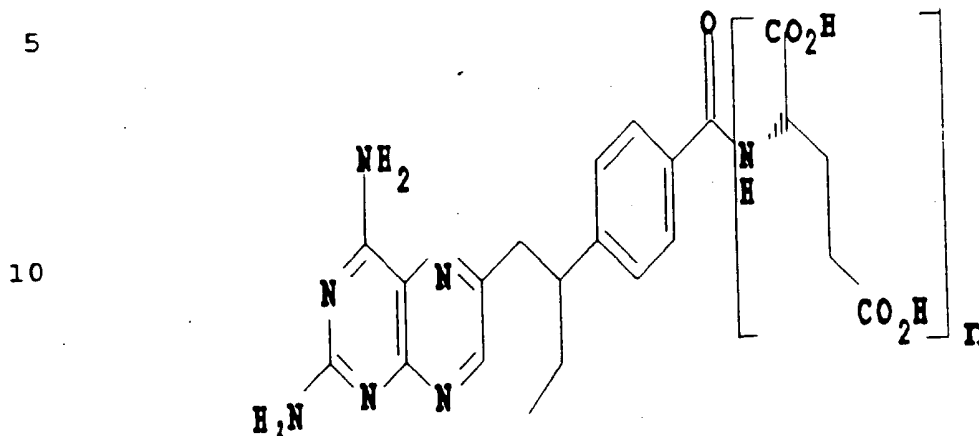


wherein n is an integer from 1-10 inclusive.

30

35

21. The compound of claim 18, wherein the compound has the structure:



wherein n is an integer from 1-10 inclusive.

22. A pharmaceutical composition comprising the compound of any of claims 18-21 in a therapeutically effective amount and a pharmaceutically acceptable carrier.
23. A method of making prostate cells susceptible to a cytotoxic chemotherapeutic agent, which comprises contacting the prostate cells with an the compound of any claims 18-21 in an amount effective to render the prostate cells susceptible to the cytotoxic chemotherapeutic agent.

PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

ABSTRACT OF THE INVENTION

5

This invention provides an isolated nucleic acid molecule encoding an alternatively spliced human prostate-specific membrane antigen. This invention provides an isolated nucleic acid comprising a promoter
10 sequence normally associated with the transcription of a gene encoding a human prostate-specific membrane antigen. This invention provides an isolated polypeptide having the biological activity of an alternatively spliced prostate-specific membrane
15 antigen.

This invention provides a method of detecting a nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen and a method of detecting a
20 prostate tumor cell in a subject.

Lastly, this invention provides a pharmaceutical composition comprising a compound in a therapeutically effective amount and a pharmaceutically acceptable
25 carrier and a method of making prostate cells susceptible to a cytotoxic chemotherapeutic agent.

1/102

FIGURE 1A

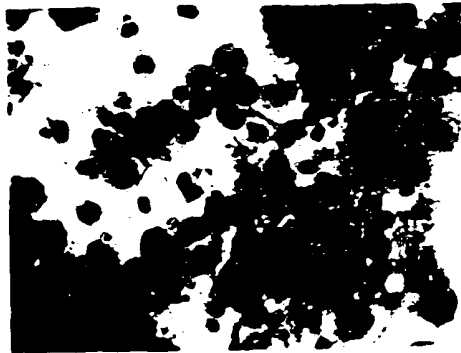


FIGURE 1B

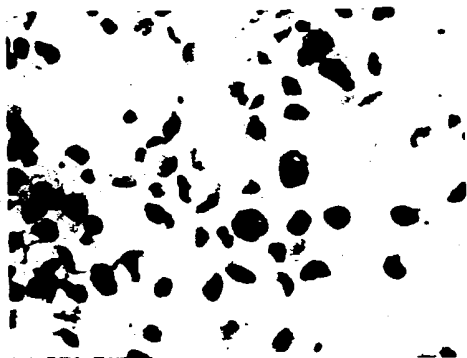


FIGURE 1C



2/102

FIGURE 2

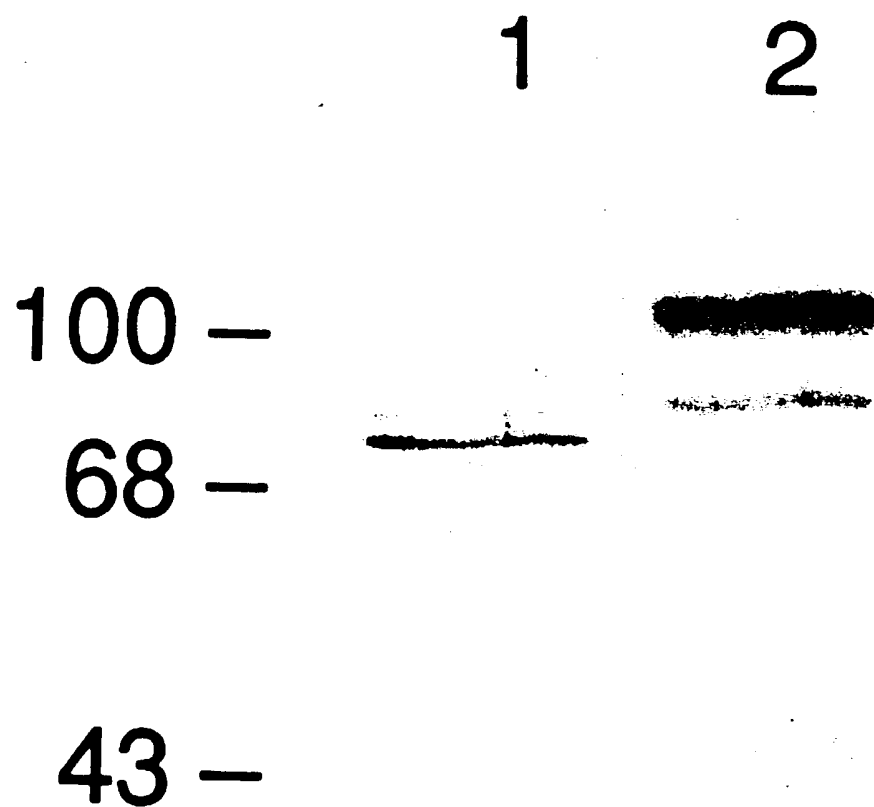
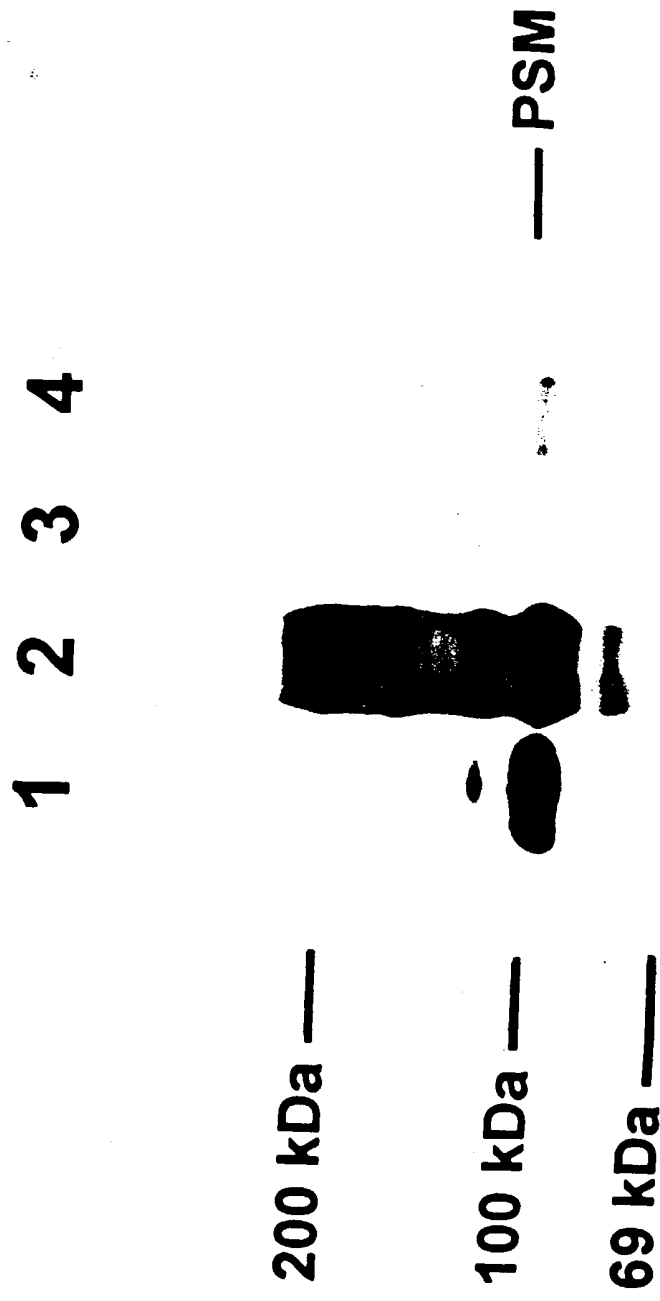


FIGURE 3



3/102

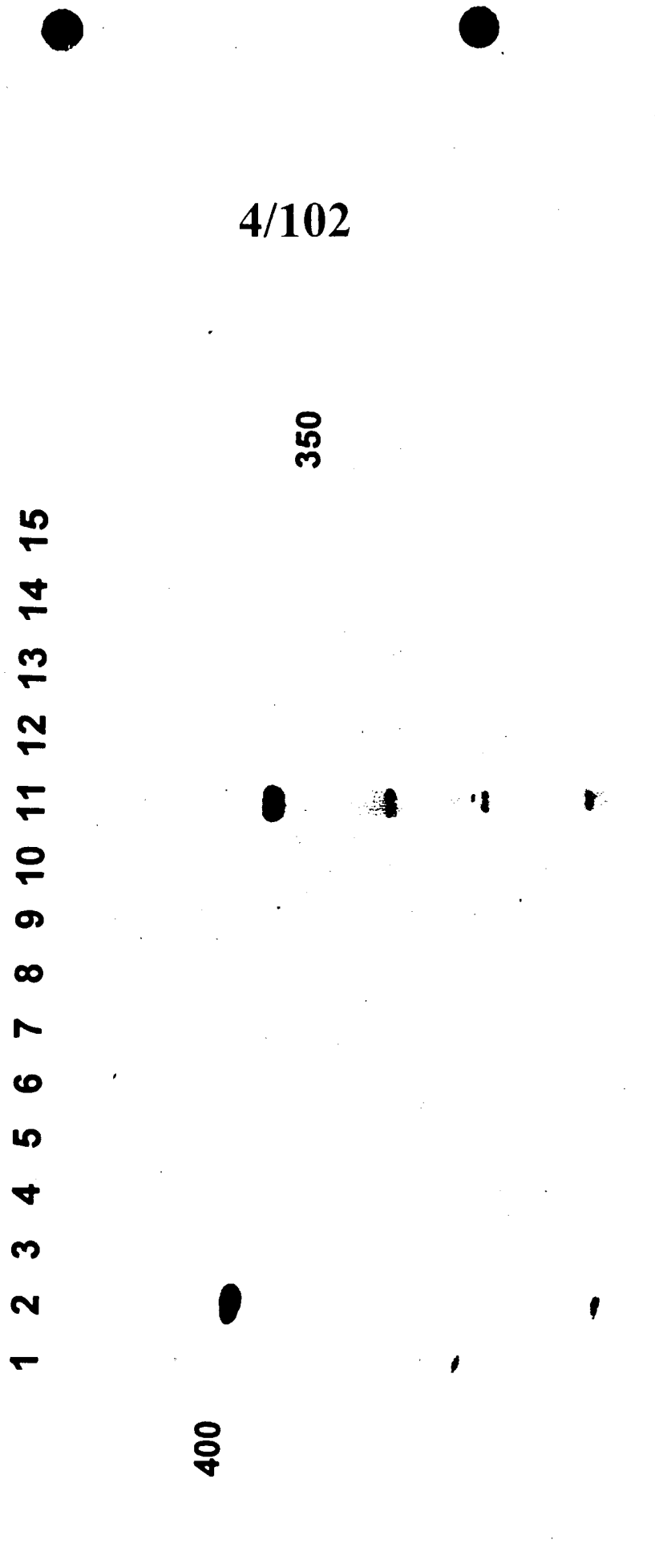
4/102

350

FIGURE 4

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

400



5/102

FIGURE 5

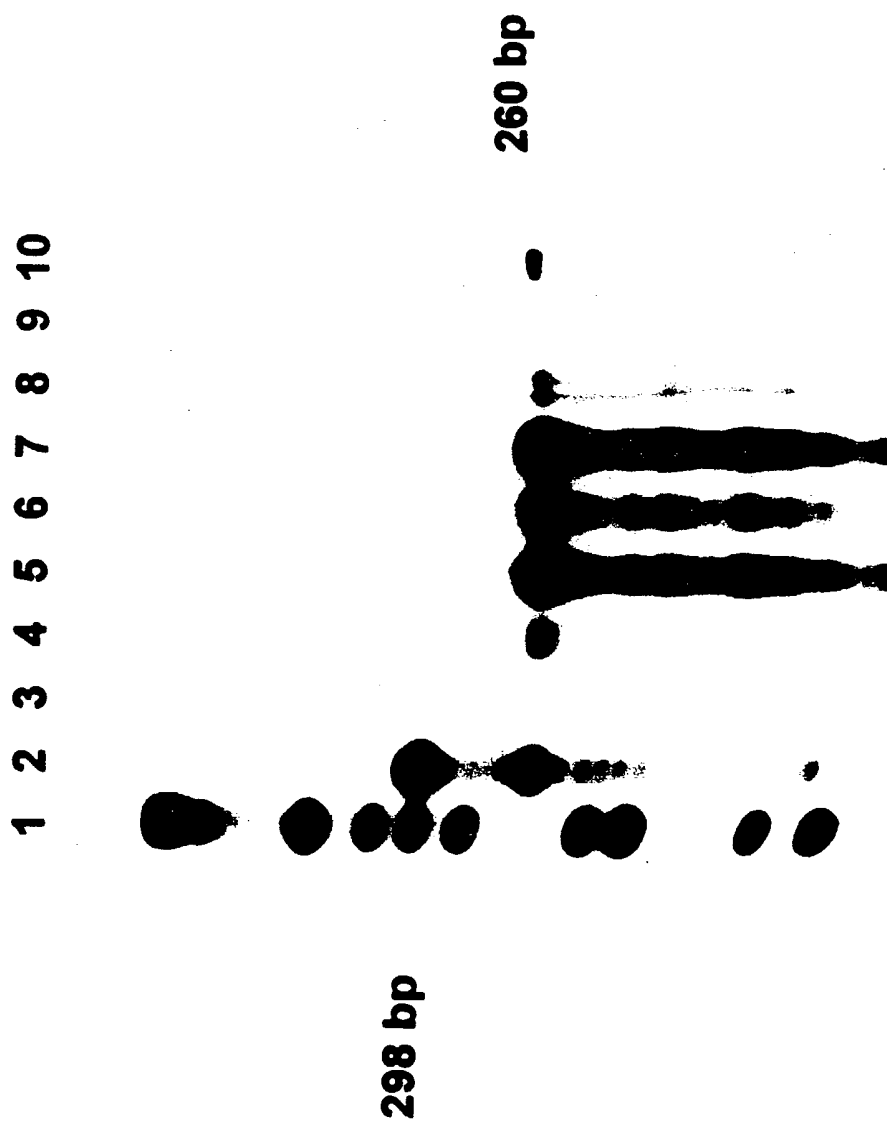


FIGURE 6

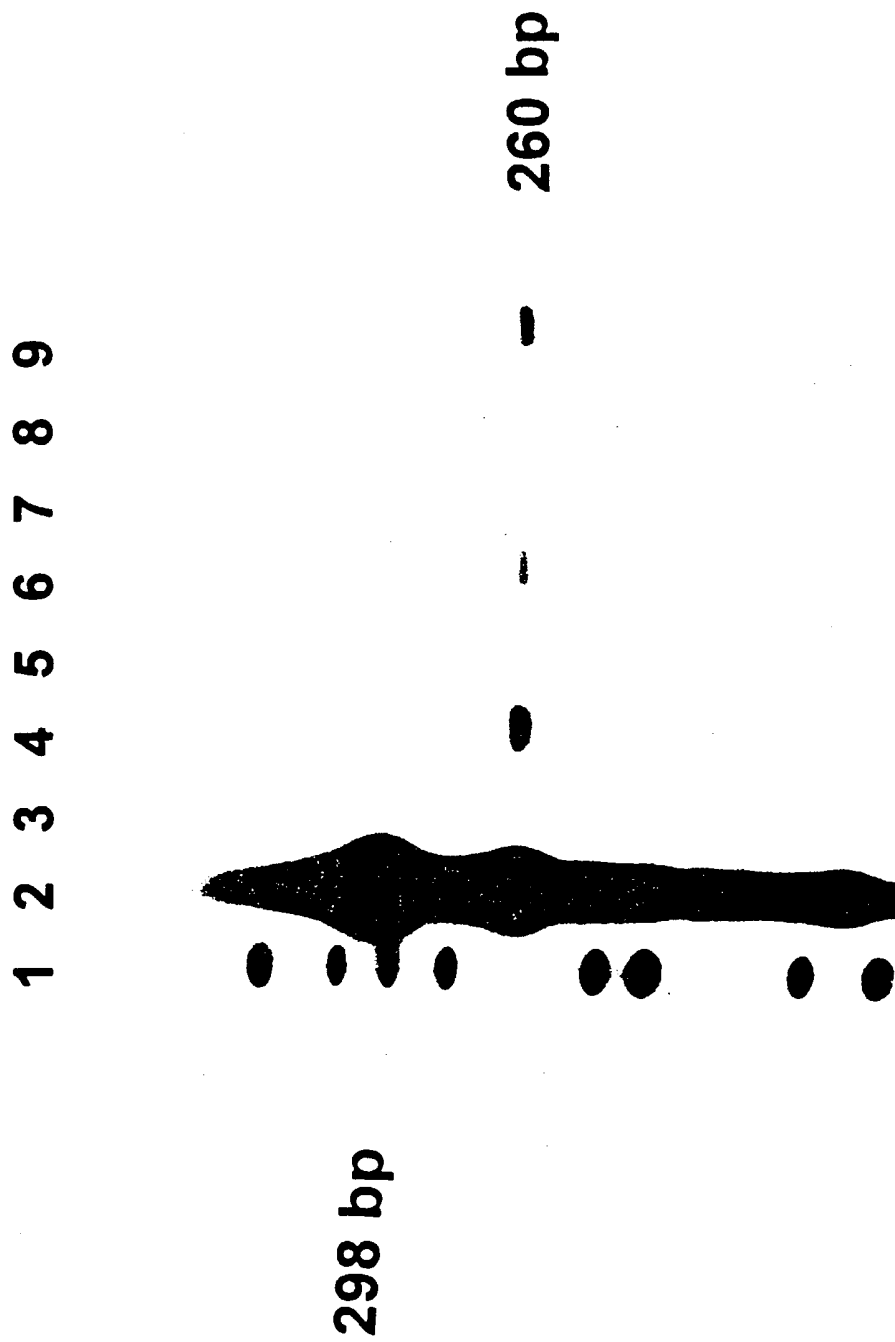


FIGURE 7

CELL LINE/TYPE	11p11.2-13 REGION	METASTATIC	PSM RNA DETECTED	PSM DNA DETECTED
LNcap			++	ND
HUMAN PROSTATE			++	ND
A9 (FIBROSARCOMA)	NO	NO	-	-
A9(11) (A9+HUM. 11)	YES	NO	-	REPEAT
AT6.1 (RAT PROSTATE)	NO	YES	-	-
AT6.1-11-c11	YES	NO	+	++
AT6.1-11-c12	NO	YES	-	-
R1564 (RAT MAMMARY)	NO	YES	-	-
R1564-11-c14	YES	YES	-	+
R1564-11-c15	YES	YES	-	REPEAT
R1564-11-c16	YES	YES	-	ND
R1564-11-c12	YES	YES	ND	+

8/102

FIGURE 8A

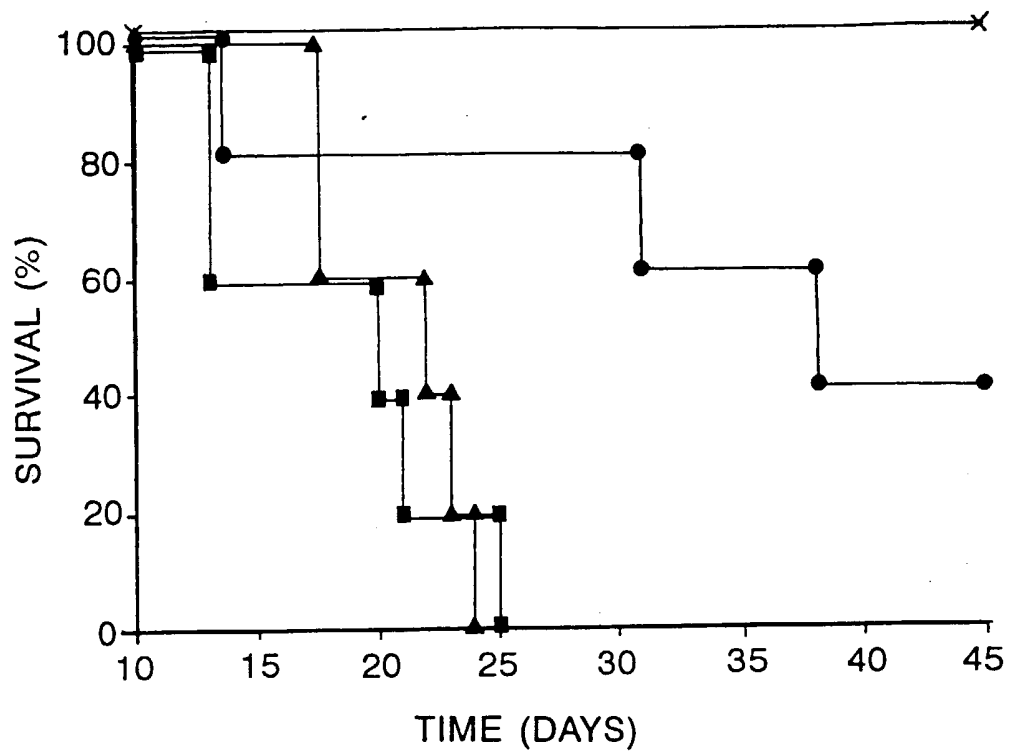


FIGURE 8B

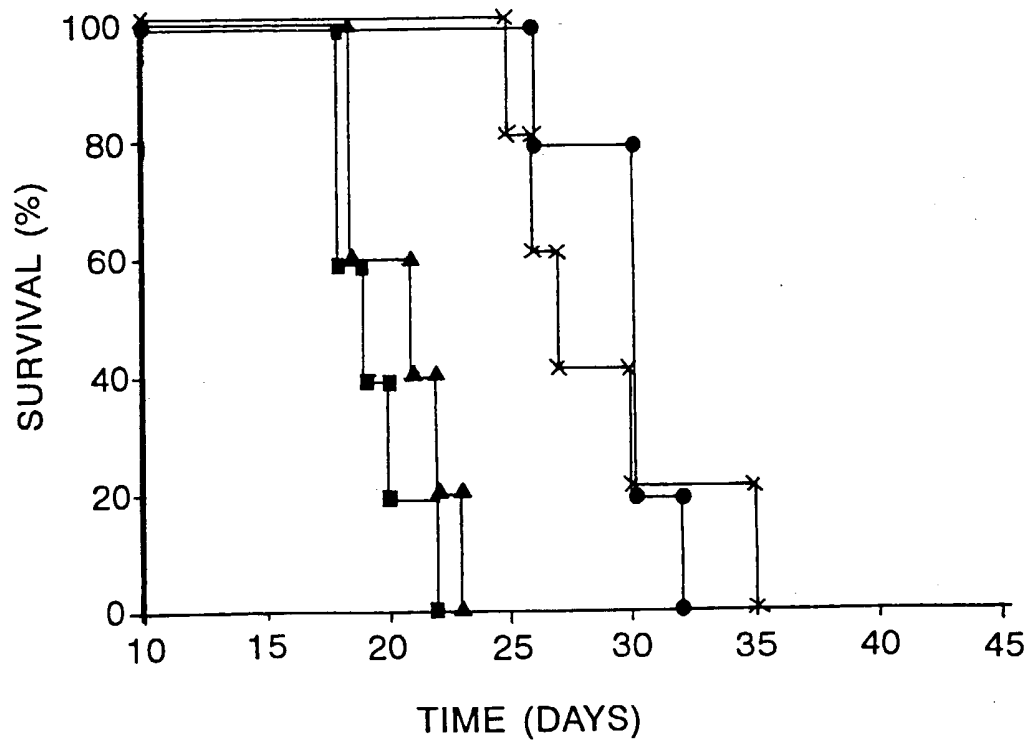


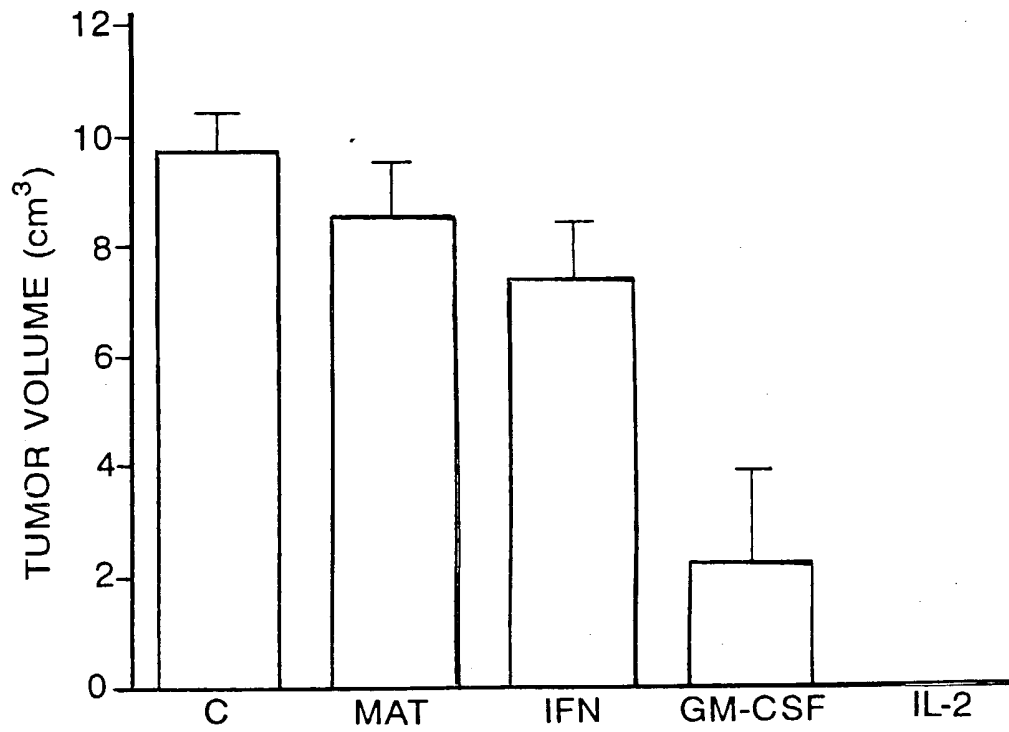
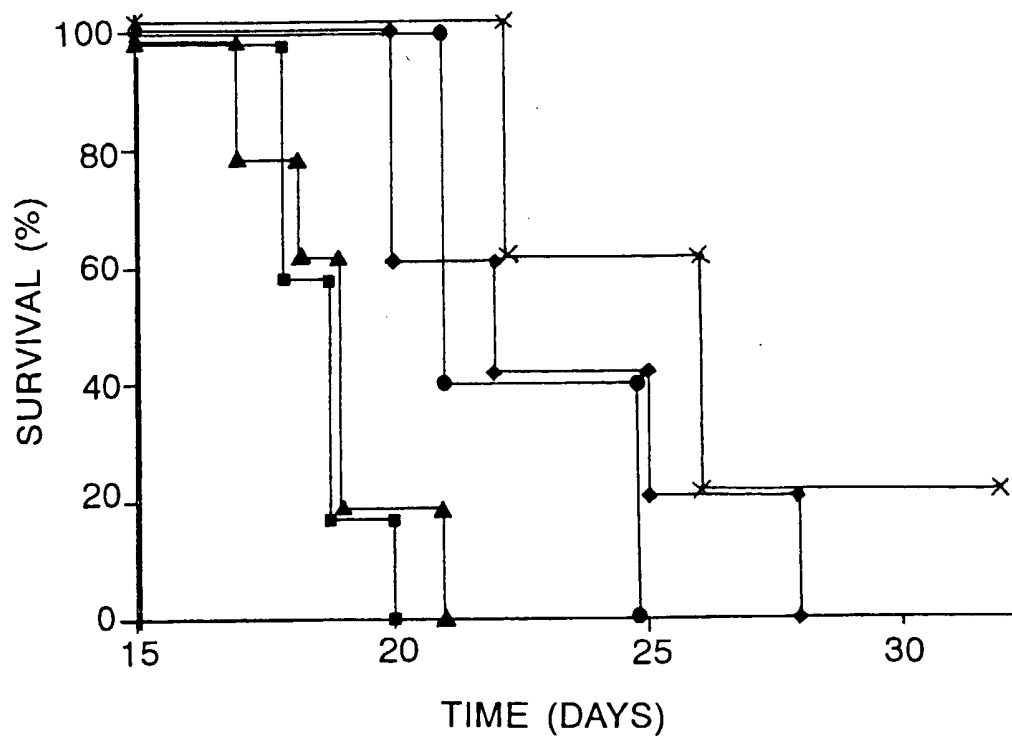
FIGURE 9A**FIGURE 9B**

FIGURE 10

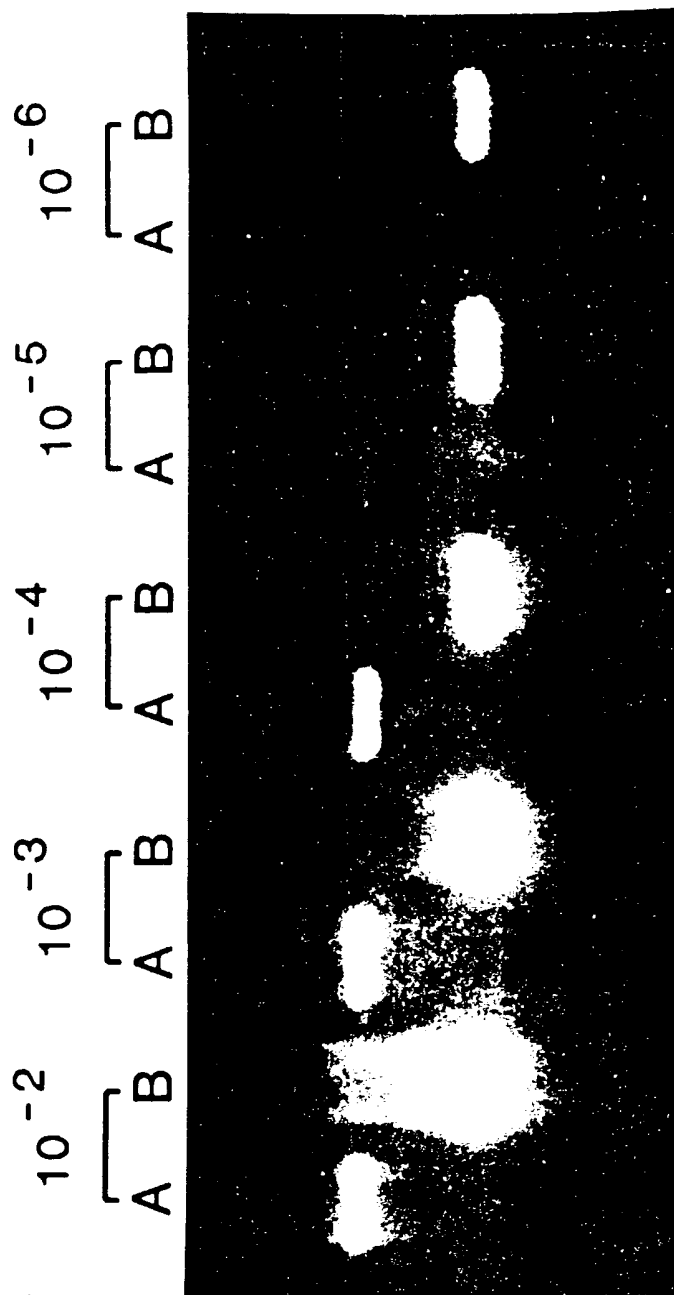


FIGURE 11

10^{-3} 10^{-4} 10^{-5} $1/10$
A B A B A B A B
M A B A B A B A B

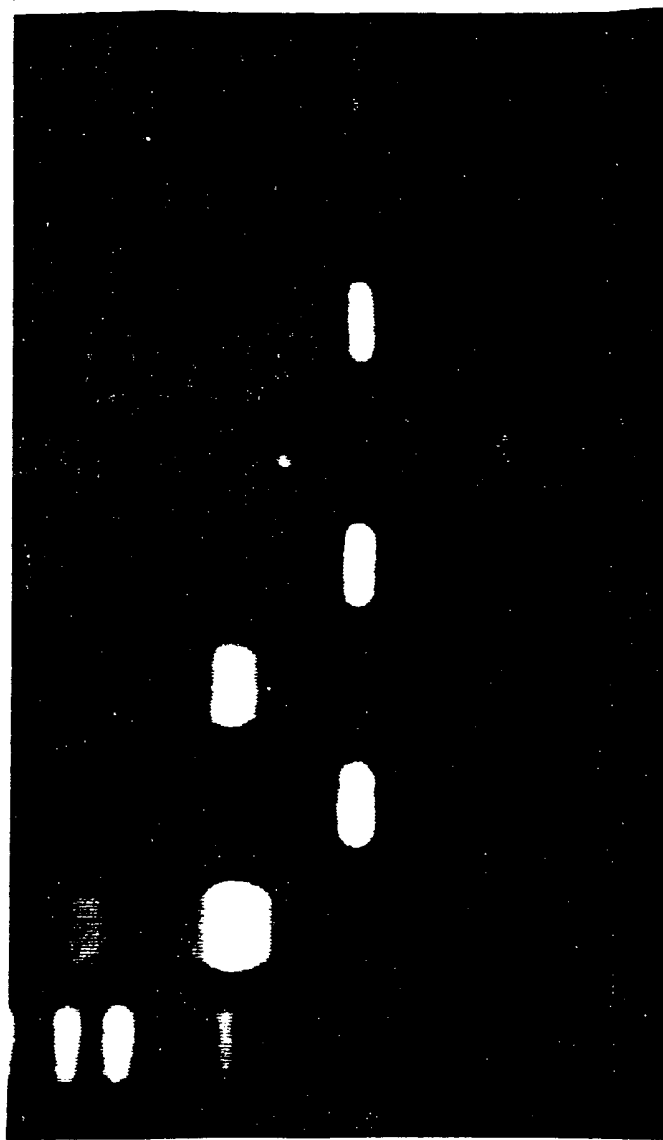


FIGURE 12

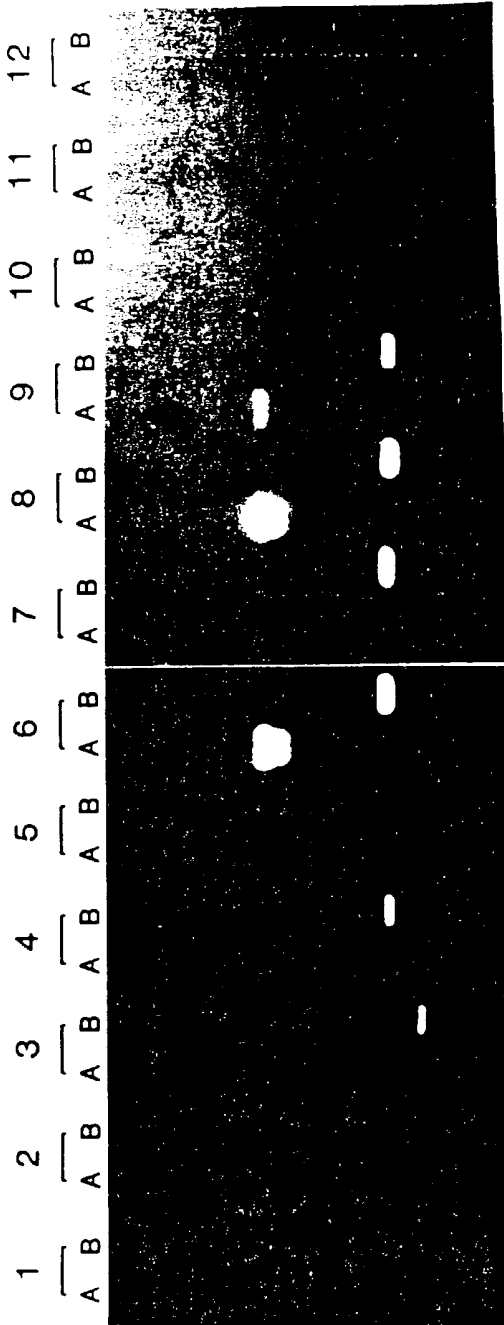


FIGURE 13

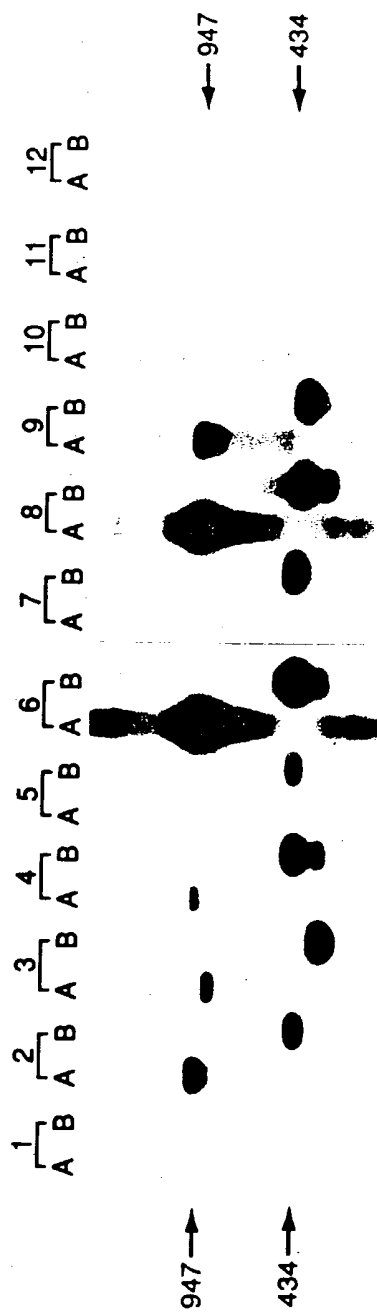


FIGURE 14

Patient	Stage	Treatment	PSA	PAP	PSA-PCR	PSM-PCR
1	T2NxMo	None	8.9	0.7	-	+
2	T2NoMo	RRP 7/93	6.1	-	-	+
3	T2CNoMo	PLND 5/93	4.5	0.1	-	+
4	T2BNoMo	RRP 3/92	NMA	0.4	-	+
5	T3NxMo	Proscar + Flutamide	51.3	1.0	-	+
6	Recur T3	I-125 1986	54.7	1.4	-	+
7	T3ANoMo	RRP 10/92	NMA	0.3	-	+
8	T3NxMo	XRT 1987	7.5	0.1	-	-
9	T3NxMo	Proscar + Flutamide	35.4	0.7	-	-
10	D2	S/P XRT Flutamide + Emcyt	311	4.5	+	+
11	D2	RRP 4/91 Lupron 10/92 Velban + Emcyt 12/92	1534	1.4	+	+
12	T2NoMo	RRP 8/91	NMA	0.5	-	+
13	T3NoMo	RRP 1/88 Lupron + Flutamide 5/92	0.1	0.3	-	-
14	D1	PLND 1989 XRT 1989	1.6	0.4	-	-
15	D1	Proscar + Flutamide	20.8	0.5	-	-
16	T2CNoMo	RRP 4/92	0.1	0.3	-	-

FIGURE 15A

	10	20	30	40	50	60
1	GCGCCTTAAA	AAAAAAAAAAC	TTTCTTGGA	AATGTCCAGC	TCTTGCTTAA	ATATAAAAAT
	CGCGGAATTT	TTTTTTTTTG	AAAGAACCTT	TTACAGGTCG	AGAACGAATT	TATATTTTTA
61	GAAAGGAAGA	AAGAGACTCT	CCTCTCTCCA	CTCCTATAAT	TATGAGGAAC	TTTTATTCAA
	CTTCCTTCT	TTCTCTGAGA	GGAGAGAGGT	GAGGATATTA	ATACTCCTTG	AAAATAAGTT
121	CTCTGAAATT	CTATACAATC	TCTACAATAC	TCTACTGAAT	AAAAGCAGAG	CAGAAAAAGC
	GAGACTTTAA	GATATGTTAG	AGATGTTATG	AGATGACTTA	TTTTCGTCTC	GTCTTTTTTCG
181	TGCGCTTTTT	TTCCATAGTC	GGGAATGCTT	GTCATCAGTG	TAAATCACCA	CCGCGCCCTT
	ACGCGAAAAA	AAGGTATCAG	CCCTTACGAA	CAGTAGTCAC	ATTTAGTGGT	GGCGCGGGAA
241	TTTCCTAAAG	AATATTATTG	TTATTAATAA	ACATGTAGGG	TATTATCCTC	CACTTACATT
	AAAGGATTTT	TTATAATAAC	AATAATTATT	TGTACATCCC	ATAATAGGAG	GTGAATGTAA
301	ACAAAACCAT	TTTTTAAAGC	CGGGCGTGCT	GGCTCACGCC	TGTAATCCCA	GCACTTTGGG
	TGTTTTGGTA	AAAAATTTTC	GCCCGCACCA	CCGAGTGCGG	ACATTAGGGT	CGTGAAACCC
361	AGGCCAGAC	AGGCGGATCA	CGAAGTCGAG	AAATCGAGAC	CATCCTGGCC	AACATGGTGA
	TCCGGGTCTG	TCCGCCTAGT	GCTTCAGCTC	TTTAGCTCTG	GTAGGACCGG	TTGTACCACT
421	AACCCCATCT	CTACTAAAAA	TACAAAAATT	AGCTGGGCGT	GGTGGCGGGC	TCCTGTAGTC
	TTGGGGTAGA	GATGATTTTT	ATGTTTTTAA	TGCACCCGCA	CCACCGCCCC	AGGACATCAG
481	CCAGCTACTC	AGGAGGCTGA	GGCAGGAGAA	TCGCTTGAAC	CGGGGAGGCG	GAGGTTGCAG
	GGTCGATGAG	TCCTCCGACT	CCGTCCTCTT	AGCGAACTTG	GCCCCCTCCG	CTCCAACGTC
541	TCAGCCAAGA	TAGCGCCACT	GCACTGGAGC	CTGGTGACAG	AGTGAGACTC	CCTCAAGAAA
	AGTCGGTTCT	ATCGCGGTGA	CGTGACCTCG	GACCACTGTC	TCACTCTGAG	GGAGTTCTTT
601	GAAAGGAAGG	GAAGGGAAAAG	GGAAGGAAGG	GGAGGGGAAG	GGAGGGGAGG	GGAGGGGAGG
	CTTCCTTCC	CTTCCTTCC	CCTTCCTTCC	CCTCCCCTTC	CCTCCCCTCC	CCTCCCCTCC
661	AAAGAAAAGA	ATACTGGAAC	TTGTTGAAGG	CAGAGACTTT	ATTTTCATAT	CCCGGCTATG
	TTTCTTTTCT	TATGACCTTG	AACAATTCC	GTCTCTGAAA	TAAAAGTATA	GGGCCGATAC
721	TCTGGCTACT	GTCTTACGTA	ATAGATATAA	AATCAATCTT	GGTTGGATTA	ACCAGAAGAA
	AGACCGATGA	CAGAATGCAT	TATCTATATT	TTAGTTAGAA	CCAACCTAAT	TGGTCTTCTT

FIGURE 15B

781 TGAGAAGATA TATTCTGGTA AGTTGAATAC TTAGCACCCA GGGGTAATCA GCTTGGACAG
 ACTCTTCTAT ATAAGACCAT TCAACTTATG AATCGTGGT CCCCATAGT CGAACCTGTG

841 GACCAGGTCC AAAGACTGTT AAGAGTCTTC TGACTCCAAA CTCAGTGCTC CCTCCAGTGC
 CTGGTCCAGG TTTCTGACAA TTCTCAGAAG ACTGAGGTTT GAGTCACGAG GGAGSTCACG

901 CACAAGCAAA CTCCATAAAG GTATCCTGTG CTGAATAGAG ACTGTAGAGT GGTACAAAAGT
 GTGTTCTGTTT GAGGTATTTT CATAGGACAC GACTTATCTC TGACATCTCA CCATCTTTCA

961 AAGACAGACA TTATATTAAG TCTTAGCTTT GTGACTTCGA ATGACTTACC TAATCTAGCT
 TTCTGTCTGT AATATAATTC AGAATCGAAA CACTGAAGCT TACTGAATGG ATTAGATCGA

1021 AAATTCAGT TTEACCATGT GTAAATCAGG AAGAGTAATA GAACAAACCT TGAAGGGTCC
 TTTAAAGTCA AAATGGTACA CATTAGTCC TTCTCATTAT CTTGTTTGA ACTTCCCAGG

1081 CAATGGTGAT TAAATGAGGT GATGTACATA ACATGCATCA CTCATAATAA GTGCTCTTTA
 GTTACCACTA ATTTACTCCA CTACATGTAT TGTACGTAGT GAGTATTATT CACGAGAAAT

1141 AATATTAGTC ACTATTATTA GCCATCTCTG ATTAGATTTG ACAATAGGAA CATTAGGAAA
 TTATAATCAG TGATAATAAT CGGTAGAGAC TAATCTAAAC TGTATCTCTT GTAATCTTTT

1201 GATATAGTAC ATTCAGGATT TTGTTAGAAA GAGATGAAGA AATCCCTTC CTTCCTGCCC
 CTATATCATG TAAGTCCTAA AACAACTCTT CTCTACTTCT TTAAGGGGAAG GAAGGACGGG

1261 TAGGTCACTCT AGGAGTTGTC ATGGTTCATT GTTGACAAAT TAATTTTCCC AAATTTTCA
 ATCCAGTAGA TCCTCAACAG TACCAAGTAA CAACTGTTTA ATTAAGGCGG TTTAAAAAGT

1321 CTTTGCTCAG AAAGTCTACA TCGAAGCACC CAAGACTGTA CAATCTAGTC CATCTTTTTC
 GAAACGAGTC TTTAGATGT AGCTTCGTG GTTCTGACAT GTTAGATCAG GTAGAAAAAG

1381 CACTTAACTC ATACTGTGCT CTCCCTTTCT CAAAGCAAAC TGTTTGCTAT TCCTTGAATA
 GTGAATTGAG TATGACACGA GAGGGAAGA GTTTCGTTTG ACAAACGATA AGGAACCTAT

1441 CACTCTGAGT TTTCTGCCTT TGCCTACTCA GCTGGCCCAT GGGCCCTAAT GTTCTTCTC
 GTGAGACTCA AAAGACGGAA ACGGATGAGT CGACCGGTA CCGGGGATTA CAAAGAAGAG

1501 ATCTCCACTG GGTCAAATCC TACCTGTACC TTATGGTCT GTTAAAGCA GTGCTTCCAT
 TAGAGGTGAC CCAGTTTAGG ATGGACATGG AATACCAAGA CAATTTCTGT CACGAAGGTA

1561 AAAGTACTCC TAGCAAATGC ACGGCCTCTC TCACGGATTA TAAGAACACA GTTTATTTTA
 TTTCAATGAGG ATCGTTTACG TGCCGGAGAG AGTGCCTAAT ATTCTGTGT CAAATAAAAAT

1621 TAAAGCATGT AGCTATTCTC TCCCTCGAAA TACGATTATT ATTATTAAGA ATTTATAGCA
 ATTTCTGTACA TCGATAAGAG AGGGAGCTTT ATGCTAATAA TAATATTCT TAAATATCGT

1681 GGGATATAAT TTTGTATGAT GATTCTTCTG GTTAATCCAA CCAAGATTGA TTTTATATCT
 CCTATATTA AAACATACTA CTAAGAAGAC CAATTAGGTT GGTCTAAT AAAATATAGA

1741 ATTACGTAAG ACAGTAGCCA GACATAGCCG GGATATGAAA ATAAAGTCTC TGCCTTCAAC
 TAATGCATTC TGTATCGGT CTGTATCGGC CCTATACCTT TATTTAGAG ACGGAAGTGG

1801 AAGTTCCAGT ATTCTTTCT TCCCTCCCT CCCCTCCCT CCCTTCCCT CCCCTTCCCT
 TTCAAGGTCA TAAGAAAAGA AAGGAGGGGA GGGGAGGGGA GGGAGGGGA GGGGAAGGAA

1861 CCCTTTCCCT TCCCTTCCCT TCTTTCTTGA GGSAGTCTCA CTCTGTACC AGGCTCCAGT
 GGGAAAGGGA AGGGAAGGAA AGAAAGAACT CCTCAGAGT GAGACAGTGG TCCGAGGTCA

FIGURE 15C

17/102

1921 GCACTGGCGC TATCTTGGCT GACTGCAACC TCCGCCTCCC CGGTTCAAGC GATTCTCCTG
 CGTCACCGCG ATAGAACCGA CTGACGTTGG AGGCGGAGGG GCCAAGTTCC CTAAGAGGAC

1981 CCTCAGCCTC CTGAGTAGCT GGGACTACAG GAGCCCGCCA CCACGCCACG CTAATTTTTG
 GGAGTCGGAG GACTCATCGA CCTGATGTC CTCGGGCGGT GGTGCGGGTC GATTAAAAAC

2041 TATTTTATAGT AGAGATGGGG TTTCACCATG TTGGCCAGGA TGCTCTCGAT TTCTCGACTT
 ATAAAAATCA TCTCTACCCC AAAGTGGTAC AACCGGTCTT ACCAGAGCTA AAGAGCTGAA

2101 CGTGATCCGC CTGTCTGGGC CTCCCAAAGT GCTGGGATTA CAGGCGTGAG CCACCACGCC
 GCACTAGCGG GACAGACCGG GAGGGTTTCA CGACCCTAAT GTCCGCACTC GGTGGTGGCG

2161 CGGCTTTAAA AAATGGTTTT GTAATGTAAG TGGAGGATAA TACCCTACAT GTTTATTAAT
 GCGGAAATTT TTTACCAAAA CATTACATTG ACCTCCTATT ATGGGATGTA CAAATAATTA

2221 AACATAATA TTCTTTAGGA AAAAGGGCGC GGTGGTGATT TACACTGATG ACAAGCATTC
 TTGTTATTAT AAGAAATCCT TTTTCCCGCG CCACCACTAA ATGTGACTAC TGTTCTGTAAG

2281 CCGACTATGG AAAAAAAGCG CAGCTTTTTG TGCTCTGCTT TTATTCAGTA GAGTATTGTA
 GGCTGATACC TTTTITTCGC GTCGAAAAAG ACGAGACGAA AATAAGTCAT CTCATAACAT

2341 GAGATTGTAT AGAATTTTCT AGTIGAATAA AAGTTCCTCA TAATTATAGG AGTGGAGAGA
 CTCTAACATA TCTTAAAGTC TCAACTTATT TTCAAGGAGT ATTAATATCC TCACCTCTCT

2401 GGAGAGTCTC TTTCTTCCTT TCATTTTAT ATTTAAGCAA GAGCTGGACA TTTTCCAAGA
 CCTCTCAGAG AAAGAAGGAA AGTAAAAATA TAAATTCTTT CTCGACCTGT AAAAGGTTCT

2461 AAGTTTTTTT TTTTAAAGGC GCCTCTCAAA AGGGGCCCGA TTCTCTCTC CTGGAGGCAG
 TTCAAAAAAA AAAAATTCCG CGGAGAGTTT TCCCCGCCCT AAAGGAAGAG GACCTCCGTC

2521 ATGTTGCCCTC TCTCTCTCGC TCGGATTGGT TCAGTGCACT CTAGAAACAC TGCTGTGGTG
 TACAACGGAG AGAGAGAGCG AGCCTAACCA AGTCACGTGA GATCTTTGTG ACGACACCAC

2581 GAGAAACTGG ACCCCAGGTC TGGAGCGAAT TCCAGCCTGC AGGGCTGATA AGCGAGGCAT
 CTCTTTGACC TGGGGTCCAG ACCTCGCTTA AGGTCCGACG TCCCGACTAT TCGCTCCGTA

2641 TAGTGAGATT GAGAGAGACT TTACCCCGCC GTGGTGTTG GAGGGCGCGC AGTAGAGCAG
 ATCACTCTAA CTCTCTCTGA AATGGGGCGG CACCACCAAC CTCCCGCGCG TCATCTCTCT

2701 CAGCACAGGC CGGGGTCCCG GGAGGCCGGC TCTGCTCGCG CCGAGATGTG GAATCTCCTT
 GTCGTGTCGG CGCCCAAGGC CCTCCGGCCG AGACGAGCGC GGCTCTACAC CTTAGAGGAA

2761 CACGAAACCG ACTCGGCTGT GGCCACCGCG CGCCGCCCGC GCTGGCTGTG CGCTGGGGCG
 GTGCTTTGGC TGAGCCGACA CCGGTGGCGC GCGGCGGGCG CGACCGACAC GCGACCCCGC

2821 CTGGTGCTGG CGGGTGCTT CTTTCTCCTC GGCTTCCTCT TCGGTAGGGG GGCGCCTCGC
 GACCACGACC GCCCACCGAA GAAAGAGGAG CCGAAGGAGA AGCCATCCCC CCGCGGAGCG

2881 GGAGCAAACC TCGGAGTCTT CCCCCTGGTG CCGCGGTGCT GGGACTCGCG GGTGAGCTGC
 CCTCGTTTGG AGCCTCAGAA GGGGCACCAAC GGCGCCACGA CCCTGAGCGC CCAGTCGACG

2941 CGAGTGGGAT CCTGTTGCTG GTCTTCCCCA GGGGCGGCGA TTAGGGTCCG GGTAATGTGG
 GCTCACCTTA GGACAACGAC CAGAAGGGGT CCCCGCCGCT AATCCCAGCC CCATTACACC

3001 GGTGAGCACC CCTCGAG
 CCACTCGTGG GGAGCTC

FIGURE 15D

- 2401 GGAGAGTCTC TTTCTTCCTT TCATTTTTAT ATTTAAGCAA GAGCTGGACA TTTTCCAAGA
CCTCTCAGAG AAAGAAGGAA AGTAAAAATA TAAATTCGTT CTCGACCTGT AAAAGGTTCT

- 2461 AAGTTTTTTT TTTTAAAGGC GCCTCTCAAA AGGGGCCGGA TTTCTTCTC CTGGAGGCAG
TTCAAAAAAA AAAAATTCCG CGGAGAGTTT TCCCCGGCCT AAAGGAAGAG GACCTCCGTC

- 2521 ATGTTGCCTC TCTCTCTCGC TCGGATTGGT TCAGTGCACT CTAGAAACAC TGCTGTGGTG
TACAACGGAG AGAGAGAGCG AGCCTAACCA AGTCACGTGA GATCTTTGTG ACGACACCAC

- 2581 GAGAAACTGG ACCCCAGGTC TGGAGCGAAT TCCAGCCTGC AGGGCTGATA AGCGAGGCAT
CTCTTTGACC TGGGGTCCAG ACCTCGCTTA AGGTCGGACG TCCCGACTAT TCGCTCCGTA

- 2641 TAGTGAGATT GAGAGAGACT TTACCCCGCC GTGGTGGTTG GAGGGCGCGC AGTAGAGCAG
ATCACTCTAA CTCTCTCTGA AATGGGSCGG CACCACCAAC CTCCCGCGCG TCATCTCGTC

- 2701 CAGCACAGGC GCGGGTCCCG GGAGGCCGGC TCTGCTCGCG CCGAGATGTG GAATCTCCTT
GTGCTGTCCG CGCCCAGGGC CCTCCGGCCC ASACGAGCGC GGCTCTACAC CTTAGAGGAA

- 2761 CACGAAACCG ACTCGGCTGT GGCCACCGCG CCGCGCCCGC GCTGGCTGTG CGCTGGGGCG
GTGCTTTGGC TGAGCCGACA CCGGTGGCGC GCGGCGGGCG CGACCGACAC GCGACCCCGC

- 2821 CTGGTGCTGG CCGGTGGCTT CTTTCTCCTC GGCTTCCTCT TCGGTAGGGG GCGGCTCGC
GACCACGACC GCCCACCGAA GAAAGAGGAG CCGAAGGAGA AGCCATCCCC CCGCGGAGCG

- 2881 GGAGCAAACC TCGGAGTCTT CCCCCTGGTG CCGCGGTGCT GGGACTCGCG GGTCAGCTGC
CTCGTTTGG AGCCTCAGAA GGGGCACCAC GGCGCCACGA CCCTGAGCGC CCAGTCGACG

- 2941 CGAGTGGGAT CCTGTTGCTG GTCTTCCCCA GGGGCGGCGA TTAGGGTCGG GGTAATGTGG
GTCACCCCTA GGACAACGAC CAGAAGGGGT CCGCGCCGCT AATCCCAGCC CCATTACACC

- 3001 GGTGAGCACC CCTCGAG
CCTCGTGG GGAGCTC

FIGURE 16

Potential binding sites on the PSM promoter*

Site	Seq	**Location	#nt matched
AP1	TKAGTCA	-1145	7/7
E2-RS	ACCNNNNNNGGT	-1940	12/12
		-1951	12/12
GHF	NNNTAAATNNN	-580	11/11
		-753	11/11
		-1340	11/11
		-1882	11/11
		-1930	11/11
		-1979	11/11
		-2001	11/11
		-2334	11/11
		-2374	11/11
		-2591	11/11
		-2620	11/11
		-2686	11/11
JVC repeat	GGGNGGRR	-1165	8/8
		-1175	8/8
		-1180	8/8
		-1185	8/8
		-1190	8/8
NFkB	GGGRHTYYHC	-961	10/10
uteroglobi	RYYWSGTG	-250	8/8
		-921	8/8
		-1104	8/8
IFN	AAWAANGAAAGGR	590	13/13

Cell 41:509 (1985)

FIGURE 17

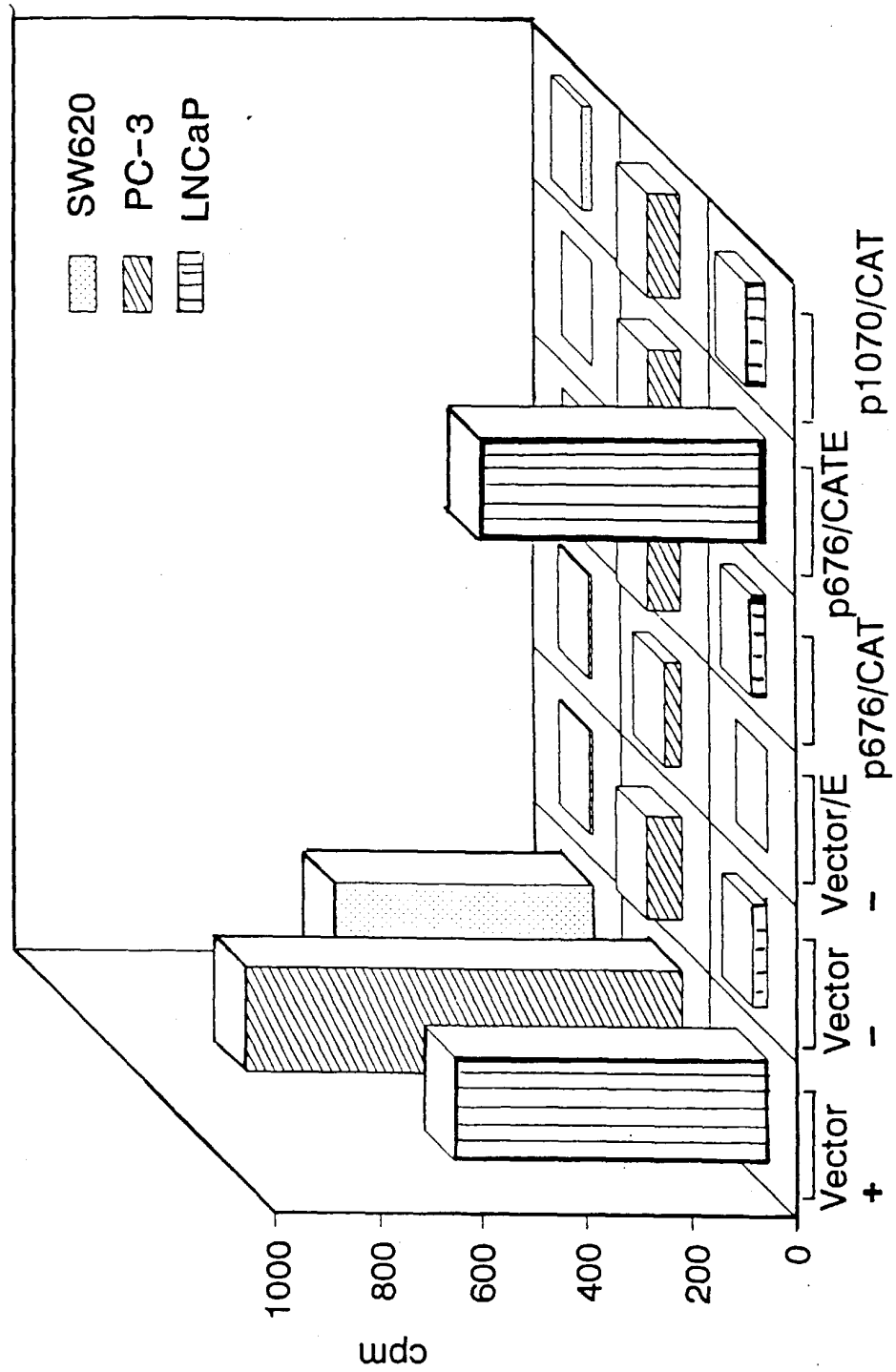


FIGURE 18

CTCAAAAGGGCCGGATTTCCT
 TCT TGGAGGCAGATGTGCCCTCTCTCTCTCGCTCGGATTGGTTCAGTGCACCTCTAGAAACACTGCTGTGGTGAGAAACT
 GGACCCC AAG TCTGGAGCGAATTCCA GCCTGCAGGGCTGATAAGCGAGGCATTAGTGAGATTGAGAGAGACTTTACCC
 CCGCGTGGTGGAGGGCGGCAGT AGAGCAGCAGCACAGGCGGGTCCCGGAGGCCGCTCTGCTCGCGCCGAG

ATG TGG AAT CTC CTT CAC GAA ACC GAC TCG GCT GTG GCC ACC GCG CCG CCG CTG CTG

Met Trp Asn Leu Leu His Glu Thr Asp Ser Ala Val Ala Ala Arg Arg Pro Arg Trp Leu

TGC GCT GGG GCG CTG GTG CTG GCG GGT GGC TTC TTT CTC CTC GGC TTC CTC TTC GGA TGG TTT

Cys Ala Gly Ala Leu Val Leu Ala Gly Gly Phe Phe Leu Leu Gly Phe Leu Phe Gly Trp Phe

ATA AAA TCC TCC AAT GAA GCT ACT AAC ATT ACT CCA AAG CAT AAT ATG AAA GCA TTT TTG GAT GAA

Ile Lys Ser Ser Asn Glu Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala Phe Leu Asp Glu

*

TGG AAA GCT GAG AAC ATC AAG AAG TTC TTA TAT AAT TTT ACA CAG ATA CCA CAT TTA GCA GGA ACA

Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile Pro His Leu Ala Gly Thr

FIGURE 19

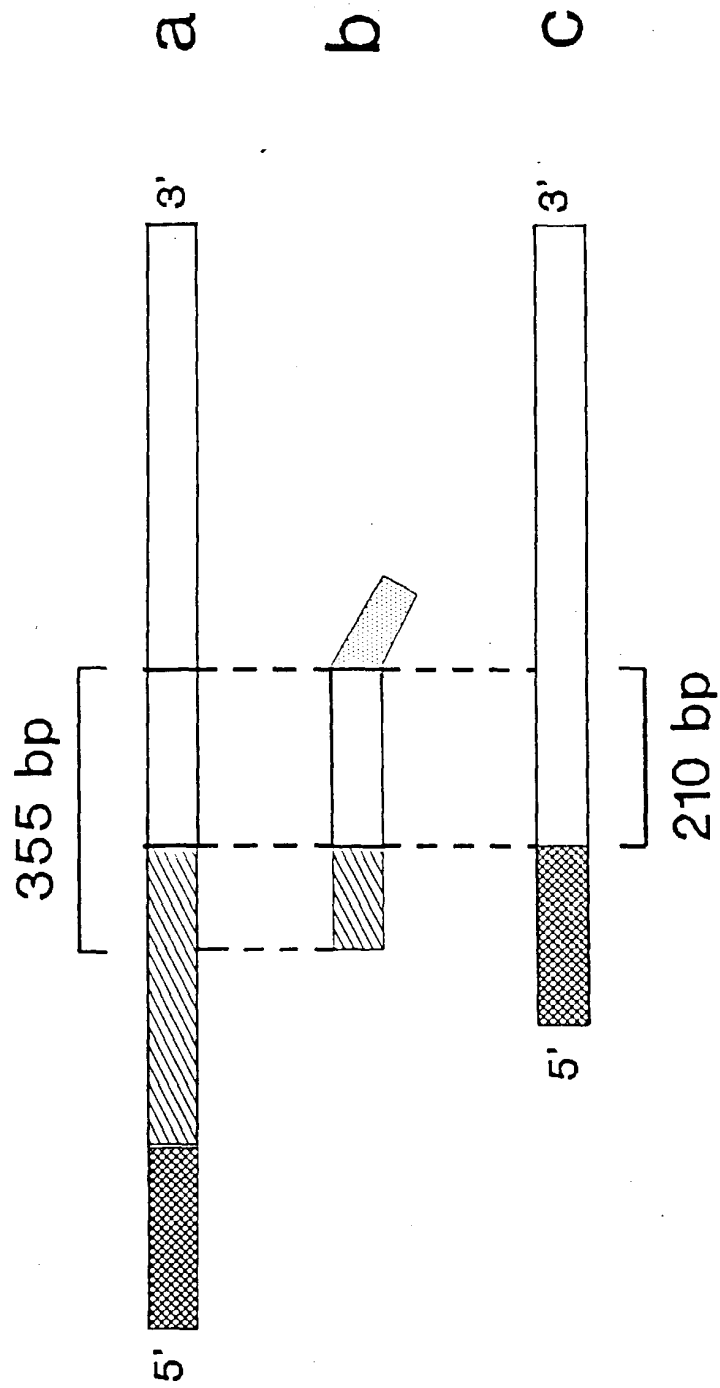


FIGURE 20

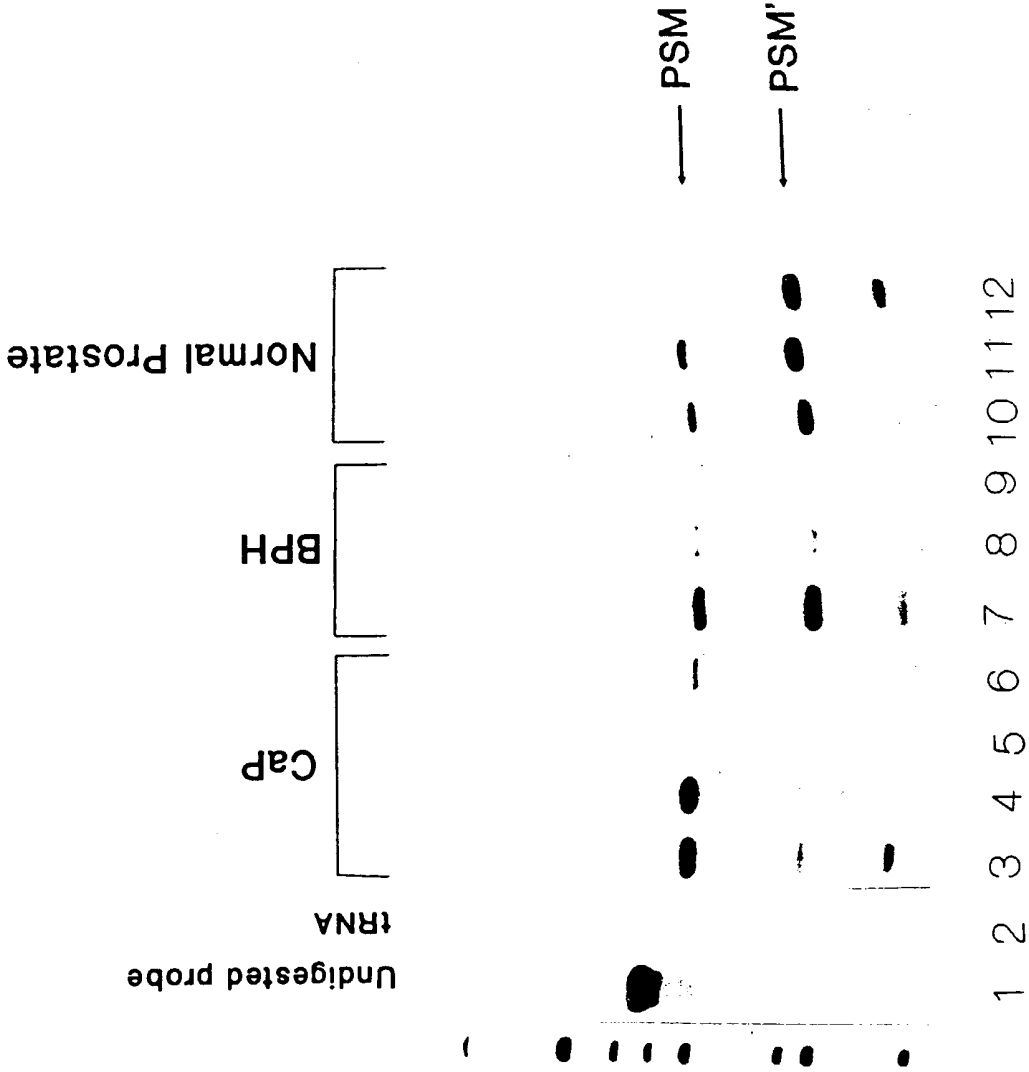
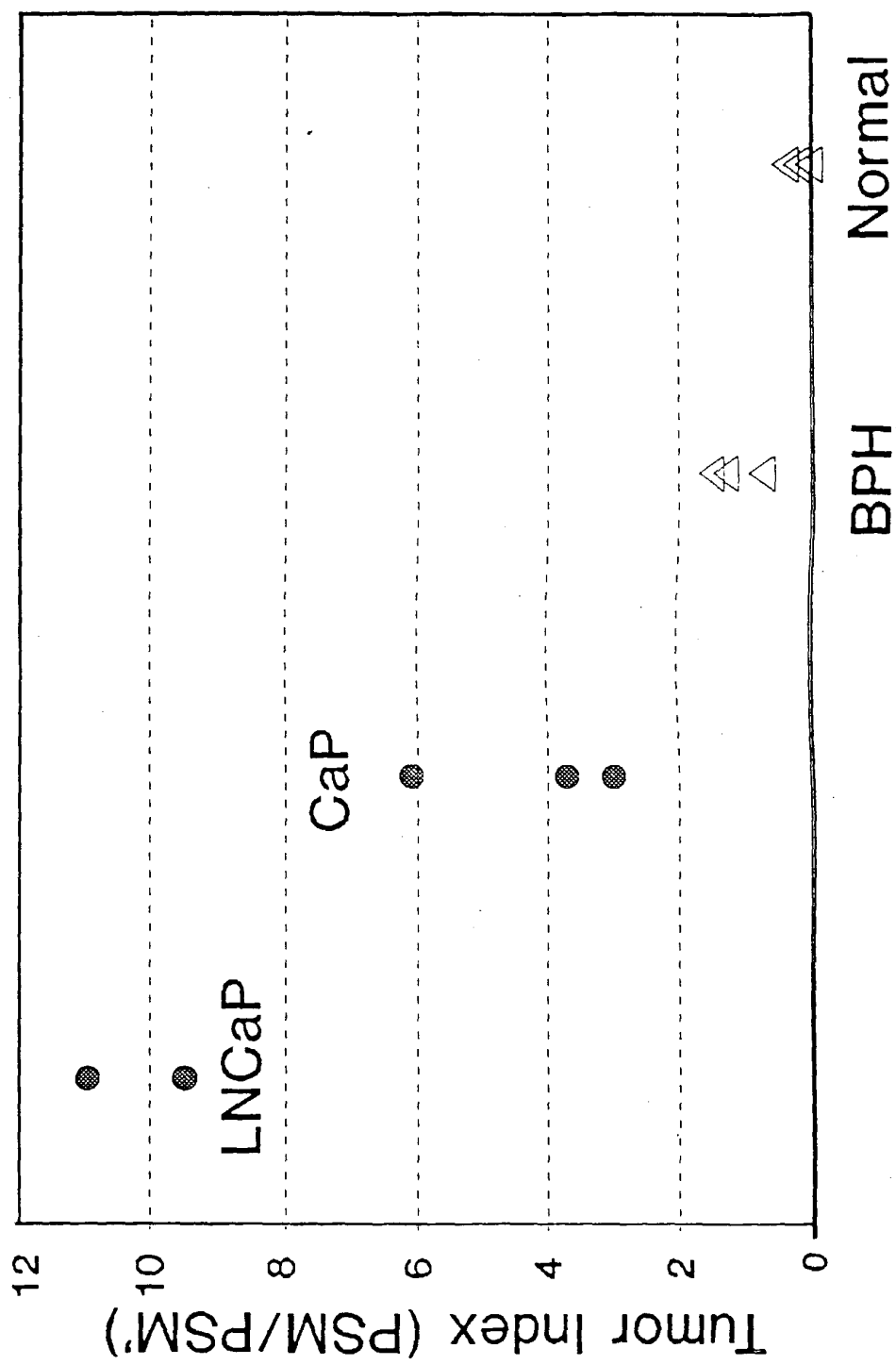


FIGURE 21



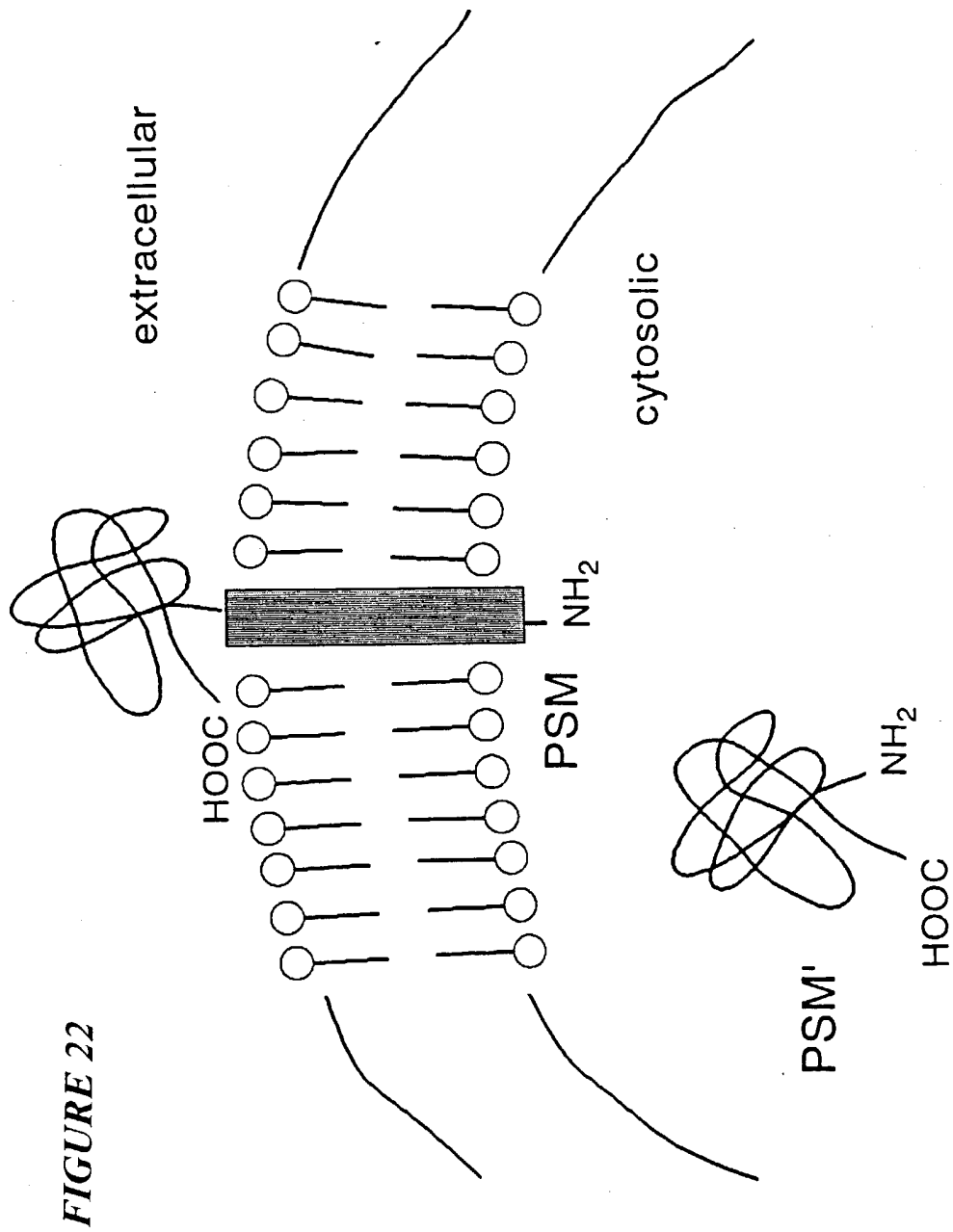


FIGURE 23

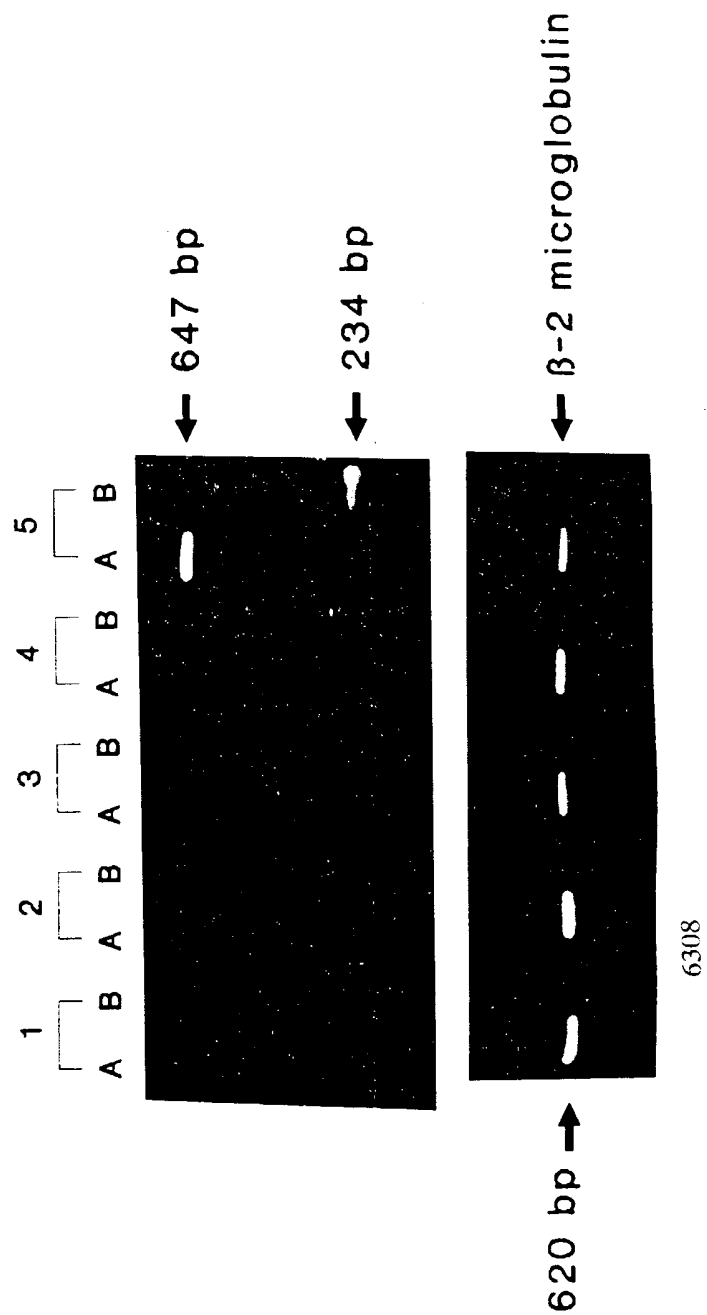


FIGURE 24

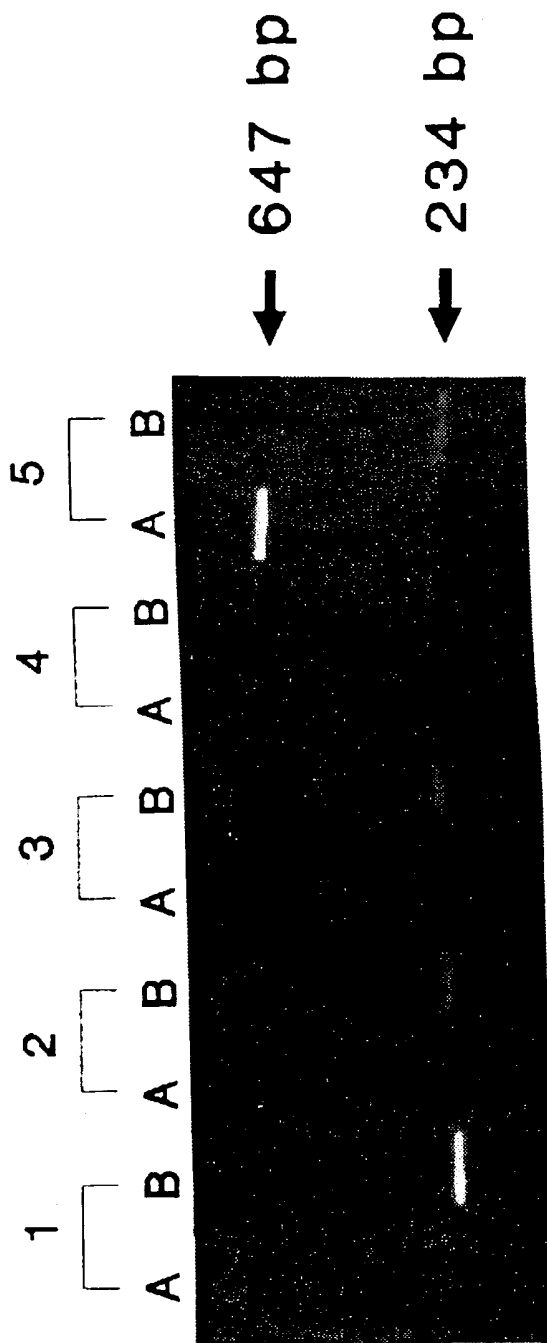
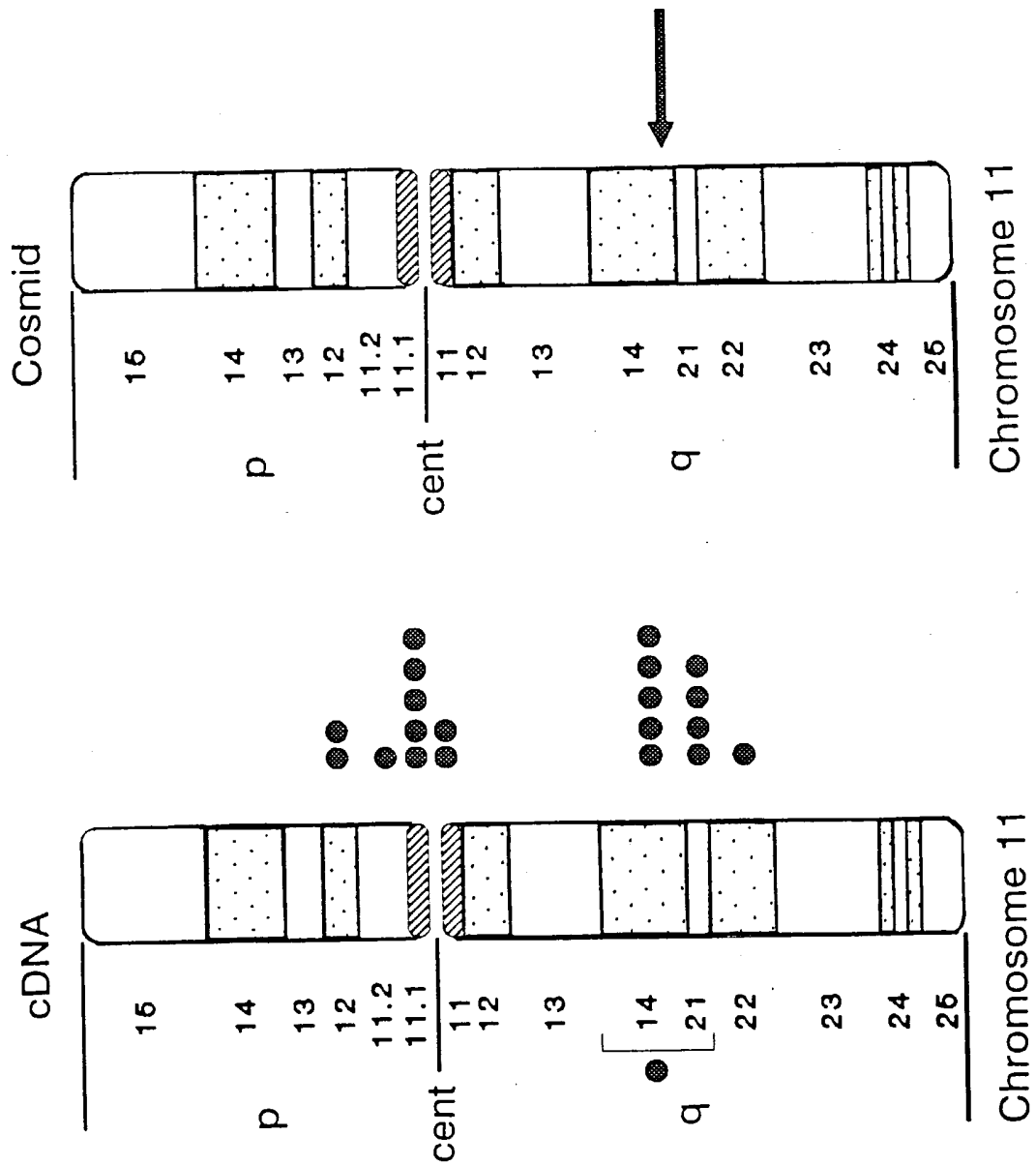


FIGURE 25



29/102

FIGURE 26

♂ ♀ M H 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y

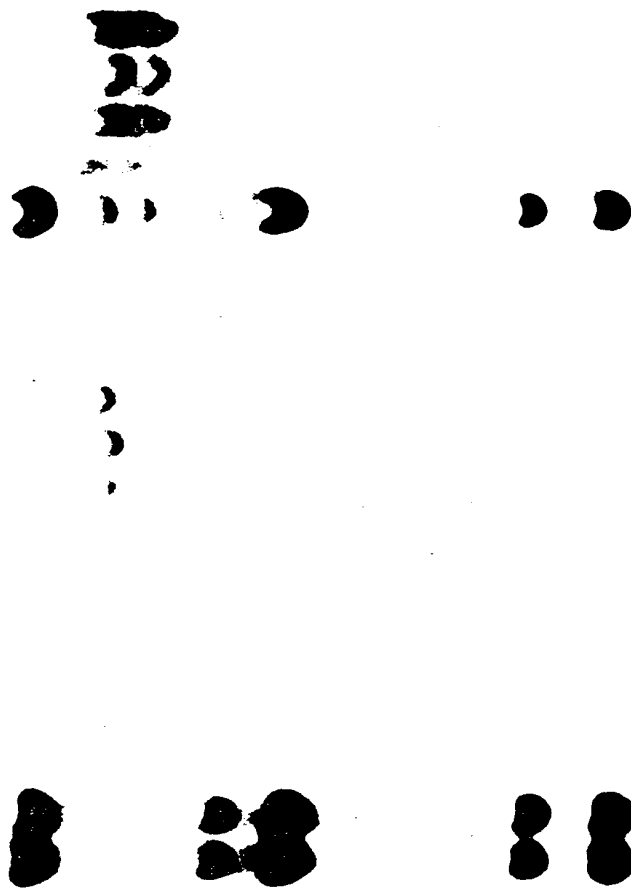
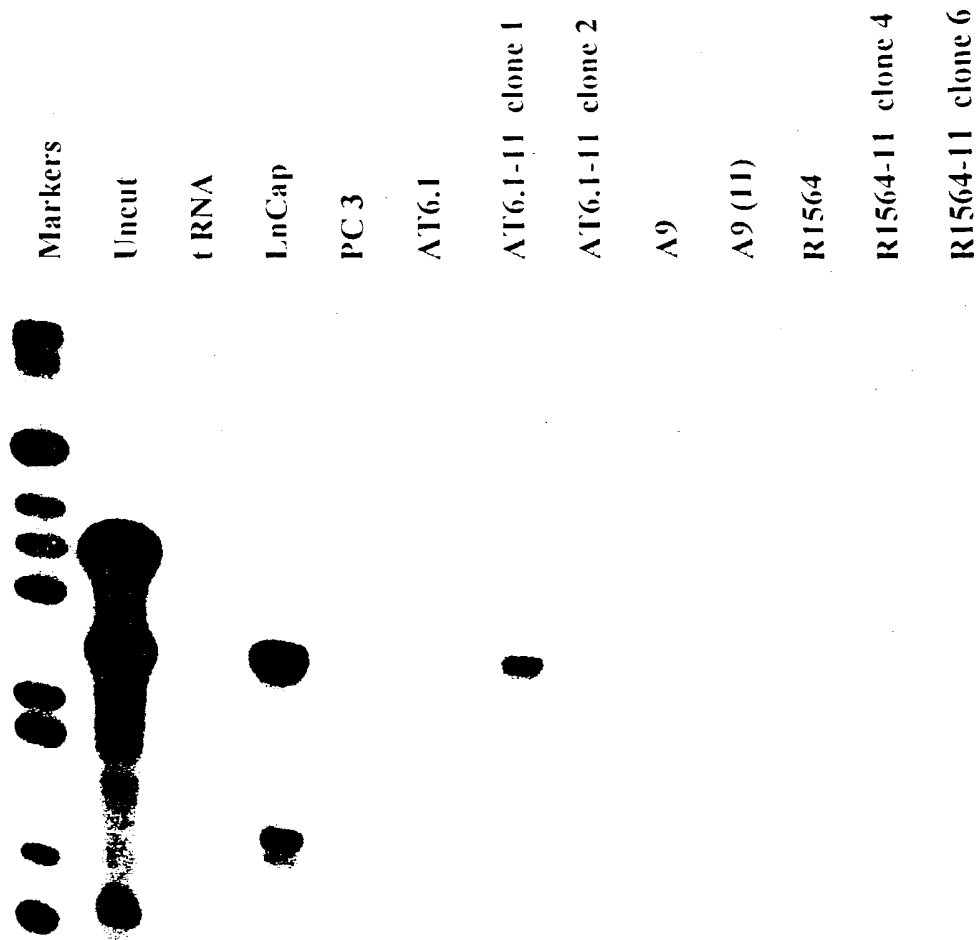


FIGURE 27



30/102

FIGURE 28

TISSUE/ CELL LINE	CANCER CELL TYPE	¹ PSM DNA	² PSM RNA
HUMAN PROSTATE	N.A.	+	+
HUMAN MAMMARY	N.A.	+	-
AT6.1	RAT PROSTATIC ADENOCARCINOMA	-	-
AT6.1-11-CL1	"	+	+
AT6.1-11-CL2	"	-	-
R1564	RAT MAMMARY ADENOCARCINOMA	-	-
R1564-11-CL2	"	+	-
R1564-11-CL4	"	+	-
R1564-11-CL5	"	+	-
R1564-11-CL6	"	+	-
A9	MOUSE FIBROSARCOMA	-	-
A9(11)	"	+	-

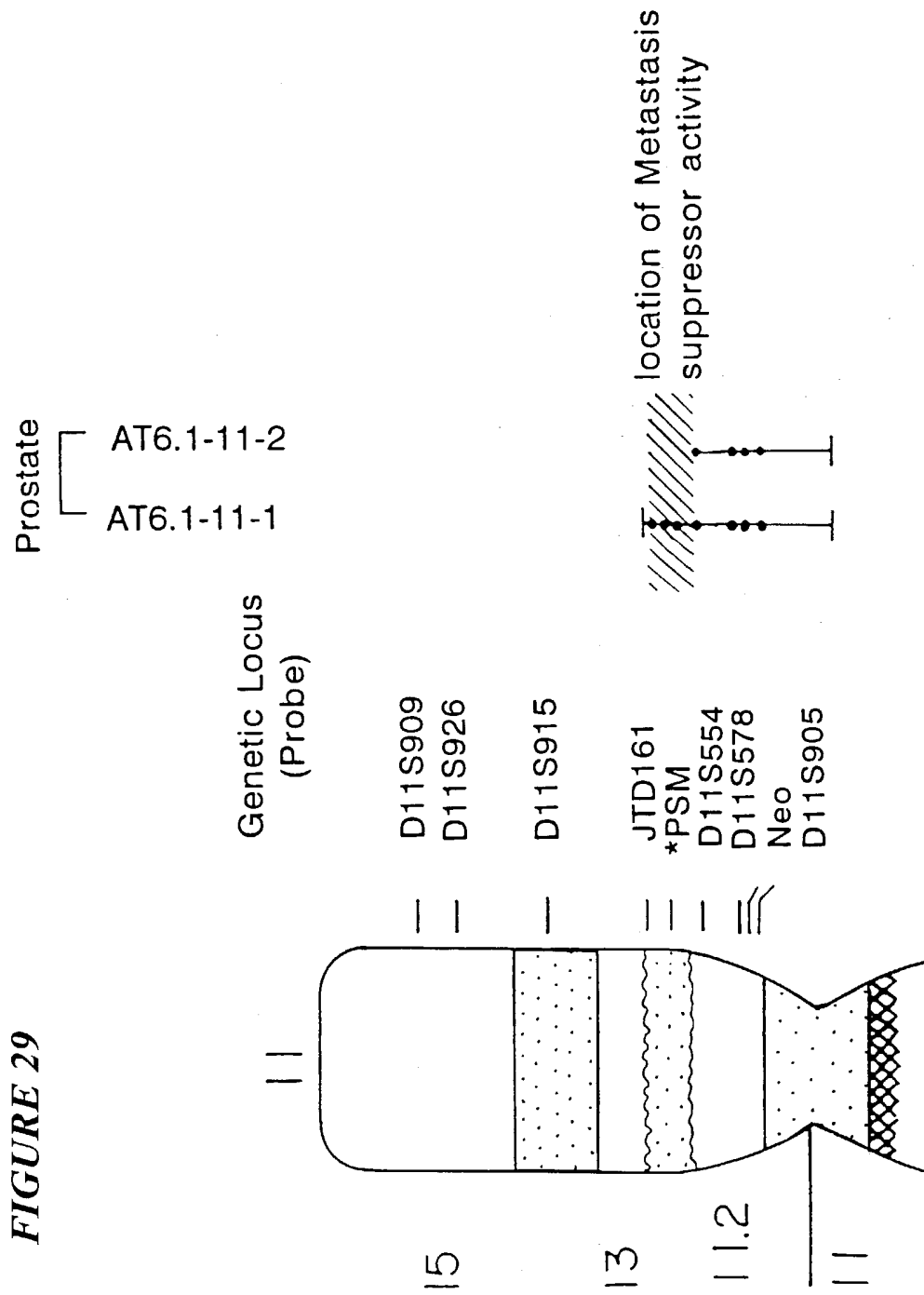


FIGURE 30

FIG. 55

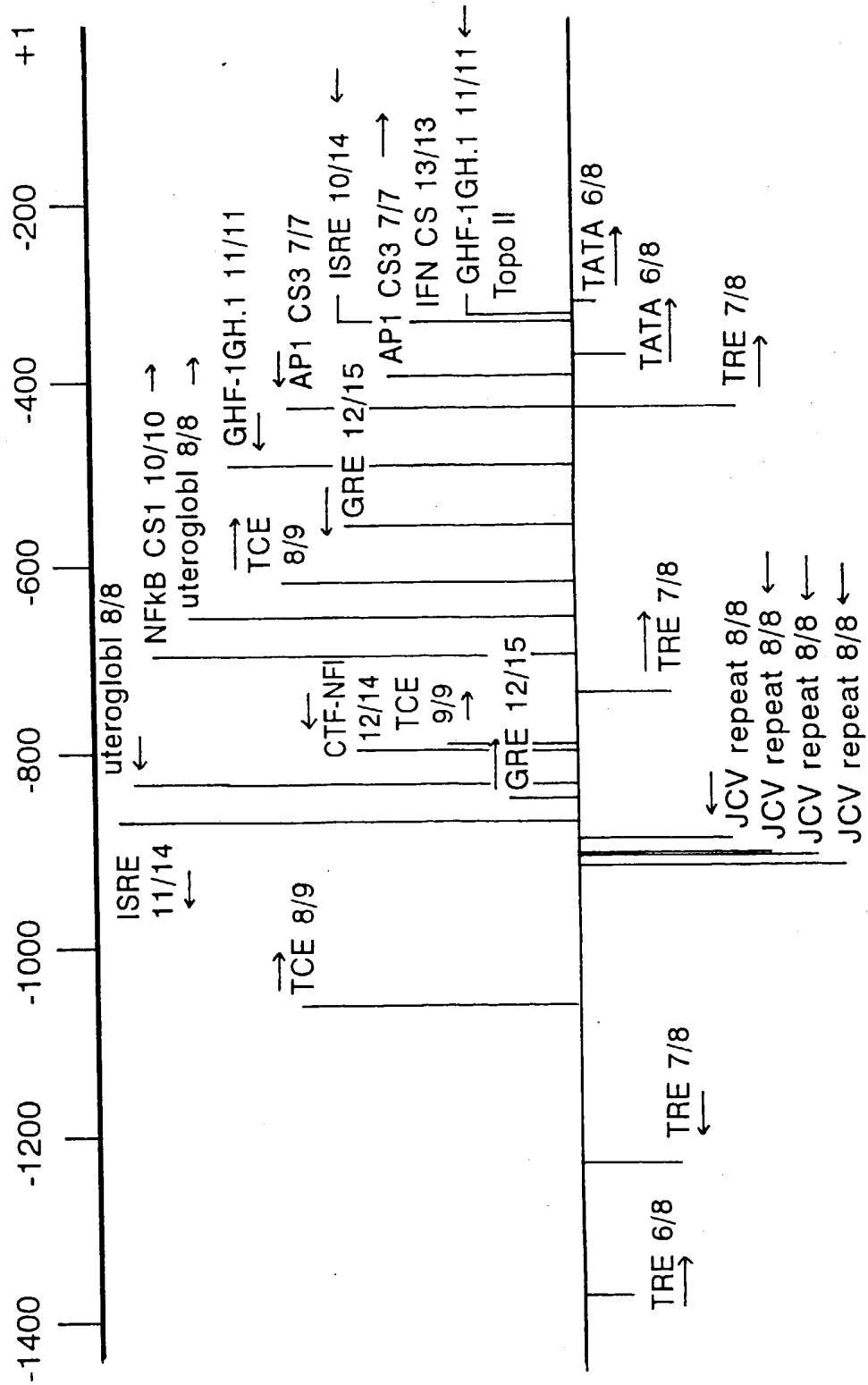


FIGURE 31

Prostate Specific Promoter: Cytosine Deaminase Chimera

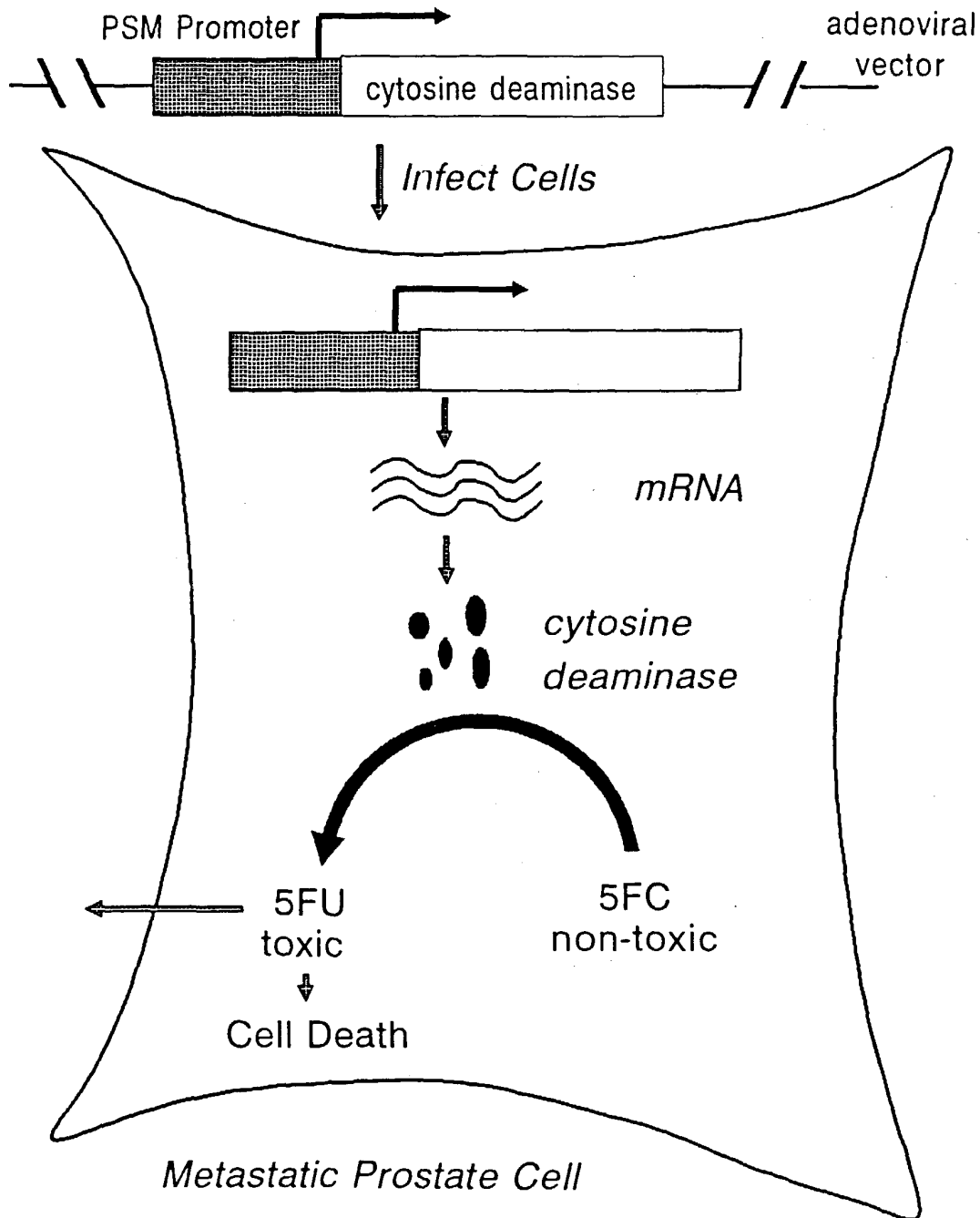


FIGURE 32A

	10	20	30	40	50	60
1	AAGGGTGCTC TTCCCACGAG	CTTAGGCTGA GAATCCGACT	ATGCTTGCAG TACGAACGTC	ACAGGATGCT TGTCTACGA	TGGTTACAGA ACCAATGTCT	TGGGCTGTGA ACCCGACACT
61	CTCGAGTGGA GAGCTCACCT	GTTTTATAAG CAAAATATTC	GGTGCTCCTT CCACGAGGAA	AGGCTGAATG TCCGACTTAC	CTTGCAGACA GAACGTCTGT	GGATGCTTGG CCTACGAACC
121	TTACAGATGG AATGTCTACC	GCTGTGAGCT CGACACTCGA	GGGTGCTTGT CCCACGAACA	AAGAGGATGC TTCTCCTACG	TTGGGTGCTA AACCCACGAT	AGTGAGCCAT TCACTCGGTA
181	TTGCAGTTGA AACGTCAACT	CCCTATTCTT GGGATAAGAA	GGAACATTCA CCTTGTAAGT	TTCCCCTCTA AAGGGGAGAT	CCCCTGTTTC GGGGACAAAG	TGTTCTGCCC ACAAGGACGG
241	AGCTAAGCCC TCGATTCTGG	ATTTTTTCATT TAAAAAGTAA	TTTCTTTTAA AAAGAAAATT	CTCCTTAGCG GAGGAATCGC	CTCCGCAAAA GAGGCGTTTT	CTTAATCAAT GAATTAGTTA
301	TTCTTTTAAAC AAGAAATTTG	CTCAGTTTTTC GAGTCAAAAAG	TTATCTGTAA AATAGACATT	AAGGTAAATA TTCCATTTAT	ATAATACAGG TATTATGTCC	GTGCAACAGA CACGTTGTCT
361	AAAATCTAGT TTTTAGATCA	GTGGTTTACA CACCAAATGT	TAATCACCTG ATTAGTGGAC	TTAGAGATTT AATCTCTAAA	TAAATTATTT ATTTAATAAA	CAGGATAAGT GTCCTATTCA
421	CATGATAATT GTACTATTAA	AAATGAAATA TTTACTTTAT	ATGCACATAA TACGTGTATT	AGCACATAGT TCGTGTATCA	GTGGTGTCTT CACCACAGGA	CCATATAGAA GGTATATCTT
481	AATGCTCAGT TTACGAGTCA	ATATTGGTTA TATAACCAAT	TTAACTACTT AATTGATGAA	GTTGAAGGTT CAACTTCCAA	TATCTTCTCC ATAGAAGAGG	ACTAAACTGT TGATTTGACA
541	AAGTTCCACA TTCAAGGTGT	AGCCTTACAA TCGGAATGTT	TATGTGACAG ATACACTGTC	ATATTCATTC TATAAGTAAG	ATTGTCTGAA TAACAGACTT	TTCTTCAAAT AAGAAGTTTA
601	ACATCCTCTT TGTAGGAGAA	CACCATAGCG GTGGTATCGC	TCTTATTAAT AGAATAATTA	TGAATTATTA ACTTAATAAT	ATTGAATAAA TAACTTATTT	TTCTATTGTT AAGATAACAA
661	CAAAAATCAC GTTTTTAGTG	TTTTATATTT AAAAATATAAA	AACTGAAATT TTGACTTTAA	TGCTTACTTA ACGAATGAAT	TAATCACATC ATTAGTGTAG	TAACCTTCAA ATTGGAAGTT
721	AGAAAACACA TCTTTTGTGT	TTAACCAACT AATTGGTTGA	GTACTGGGTA CATGACCCAT	ATGTTACTGG TACAATGACC	GTGATCCAC CACTAGGGTG	GTTTTACAAA CAAAATGTTT

FIGURE 32B

781 TGAGAAGATA TATTCTGGTA AGTTGAATAC TTAGCACCCA GGGGTAATCA GCTTGGACAG
 ACTCTTCTAT ATAAGACCAT TCAACTTATG AATCGTGGGT CCCCATTAGT CGAACCTGTC

841 GACCAGGTCC AAAGACTGTT AAGAGTCTTC TGACTCCAAA CTCAGTGCTC CCTCCAGTGC
 CTGGTCCAGG TTTCTGACAA TTCTCAGAAG ACTGAGGTTT GAGTCACGAG GGAGGTCACG

901 CACAAGCAAA CTCCATAAAG GTATCCTGTG CTGAATAGAG ACTGTAGAGT GGTACAAAGT
 TGTTTCGTTT GAGGTATTTT CATAGGACAC GACTTATCTC TGACATCTCA CCATGTTTCA

961 AAGACAGACA TTATATTAAG TCTTAGCTTT GTGACTTCGA ATGACTTACC TAATCTAGCT
 TTCTGTCTGT AATATAATTG AGAATCGAAA CACTGAAGCT TACTGAATGG ATTAGATCGA

1021 AAATTTTCAGT TTTACCATGT GTAAATCAGG AAGAGTAATA GAACAAACCT TGAAGGGTCC
 TTTAAAGTCA AAATGGTACA CATTAGTCC TTCTCATTAT CTTGTTTGGA ACTTCCCAGG

1081 CAATGGTGAT TAAATGAGGT GATGTACATA ACATGCATCA CTCATAATAA GTGCTCTTTA
 GTTACCACTA ATTTACTCCA CTACATGTAT TGTACGTAGT GAGTATTATT CACGAGAAAT

1141 AATATTAGTC ACTATTATTA GCCATCTCTG ATTAGATTTG ACAATAGGAA CATTAGGAAA
 TTATAATCAG TGATAATAAT CGGTAGAGAC TAATCTAAAC TGTATCTCTT GTAATCTTTT

1201 GATATAGTAC ATTCAGGATT TTGTTAGAAA GAGATGAAGA AATTCCCTTC CTTCTGCCC
 CTATATCATG TAAGTCCTAA AACAATCTTT CTCTACTTCT TTAAGGGAAG GAAGGACGGG

1261 TAGGTCATCT AGGAGTTGTC ATGGTTCATT GTTGACAAAT TAATTTTCCC AAATTTTTCA
 ATCCAGTAGA TCCTCAACAG TACCAAGTAA CAACTGTTTA ATTAAGGGG TTTAAAAAGT

1321 CTTTGCTCAG AAAGTCTACA TCGAAGCACC CAAGACTGTA CAATCTAGTC CATCTTTTTT
 GAAACGAGTC TTTGAGATGT AGCTTCGTGG GTTCTGACAT GTTAGATCAG GTAGAAAAAG

1381 CACTTAACTC ATACTGTGCT CTCCCTTTCT CAAAGCAAAC TGTTTGCTAT TCCTTGAATA
 GTGAATTGAG TATGACACGA GAGGGAAAGA GTTTCGTTTG ACAAACGATA AGGAACCTAT

1441 CACTCTGAGT TTTCTGCCTT TGCCTACTCA GCTGGCCCAT GGCCCTAAT GTTCTTCTC
 GTGAGACTCA AAAGACGGAA ACGGATGAGT CGACCGGTA CCGGGGATTA CAAAGAAGAG

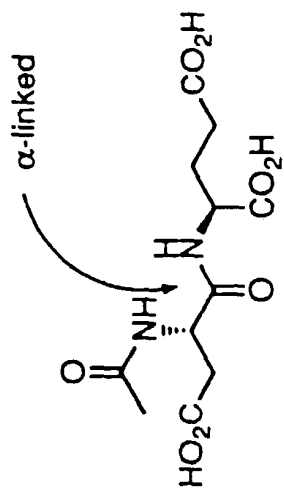
1501 ATCTCCACTG GGTCAAATCC TACCTGTACC TTATGGTTCT GTTAAAAGCA GTGCTTCCAT
 TAGAGGTGAC CCAGTTTAGG ATGGACATGG AATACCAAGA CAATTTTCGT CACGAAGGTA

1561 AAAGTACTCC TAGCAAATGC ACGGCCTCTC TCACGGATTA TAAGAACACA GTTTATTTTA

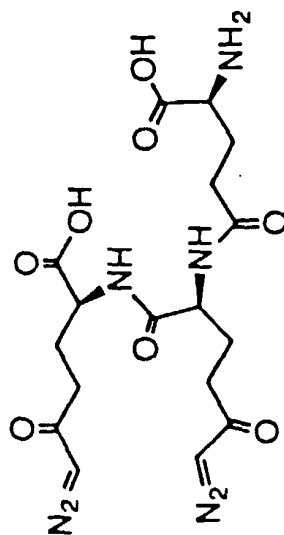
FIGURE 32C

TTTCATGAGG ATCGTTTACG TGCCGGAGAG AGTGCCTAAT ATTCTTGTGT CAAATAAAAT
 1621 TAAAGCATGT AGCTATTCTC TCCCTCGAAA TACGATTATT ATTATTAAGA ATTTATAGCA
 ATTTTCGTACA TCGATAAGAG AGGGAGCTTT ATGCTAATAA TAATAATTCT TAAATATCGT
 1681 GGGATATAAT TTTGTATGAT GATTCTTCTG GTTAATCCAA CCAAGATTGA TTTTATATCT
 CCCTATATTA AAACATACTA CTAAGAAGAC CAATTAGGTT GGTTCTAACT AAAATATAGA
 1741 ATTACGTAAG ACAGTAGCCA GACATAGCCG GGATATGAAA ATAAAGTCTC TGCCTTCAAC
 TAATGCATTC TGTCAATCGG CTGTATCGGC CCTATACTTT TATTTTCAGAG ACGGAAGTTG
 1801 AAGTTCCAGT ATTCTTTTCT TTCCTCCCCT CCCCTCCCCT CCCTTCCCCT CCCCTTCCTT
 TTCAAGGTCA TAAGAAAAGA AAGGAGGGGA GGGGAGGGGA GGGAAGGGGA GGGGAAGGAA
 1861 CCCTTTCCCCT TCCCTTCCTT TCTTTCTTGA GGGAGTCTCA CTCTGTCACC AGGCTCCAGT
 GGGAAAGGGA AGGGAAGGAA AGAAAGAACT CCCTCAGAGT GAGACAGTGG TCCGAGGTCA
 1921 GCAGTGGCGC TATCTTGGCT GACTGCAACC TCCGCCTCCC CGGTTCAAGC GATTCTCCTG
 CGTCACCGCG ATAGAACCGA CTGACGTTGG AGGCGGAGGG GCCAAGTTCC CTAAGAGGAC
 1981 CCTCAGCCTC CTGAGTAGCT GGGACTACAG GAGCCCGCCA CCACGCCCAG CTAATTTTTG
 GGAGTCGGAG GACTCATCGA CCCTGATGTC CTCGGGCGGT GGTGCGGGTC GATTAAAAAC
 2041 TATTTTLAGT AGAGATGGGG TTTCACCATG TTGGCCAGGA TGGTCTCGAT TTCTCGACTT
 ATAAAAATCA TCTCTACCCC AAAGTGGTAC AACCAGTCTT ACCAGAGCTA AAGAGCTGAA
 2101 CGTGATCCGC CTGTCTGGGC CTCCCAAAGT GCTGGGATTA CAGGCGTGAG CCACCACGCC
 GCACTAGGCG GACAGACCCG GAGGGTTTCA CGACCCCTAAT GTCCGCACTC GGTGGTGCGG
 2161 CGGCTTTAAA AAATGGTTTT GTAATGTAAG TGGAGGATAA TACCCTACAT GTTTATTAAT
 GCCGAAATTT TTTACCAAAA CATTACATTC ACCTCCTATT ATGGGATGTA CAAATAATTA
 2221 AACAATAATA TTCTTTAGGA AAAAGGGCGC GGTGGTGATT TACACTGATG ACAAGCATTC
 TTGTTATTAT AAGAAATCCT TTTTCCCGCG CCACCACTAA ATGTGACTAC TGTTTCGTAAG
 2281 CCGACTATGG AAAAAAAGCG CAGCTTTTTT TGCTCTGCTT TTATTAGTA GAGTATTGTA
 GGCTGATACC TTTTTTTCGC GTCGAAAAAG ACGAGACGAA AATAAGTCAT CTCATAACAT
 2341 GAGATTGTAT AGAATTTTCT AGTTGAATAA AAGTTCCTCA TAATTATAGG AGTGGAGAGA
 CTCTAACATA TCTTAAAGTC TCAACTTATT TTCAAGGAGT ATTAATATCC TCACCTCTCT

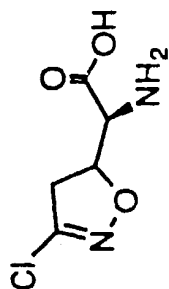
FIGURE 33



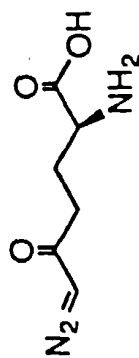
NAAG 1
N-acetylaspartyl-L-glutamate



Azotomycin, becomes active by *in vivo* conversion to DON



Activin



6-diazo-5-oxo-norleucine, DON

FIGURE 34

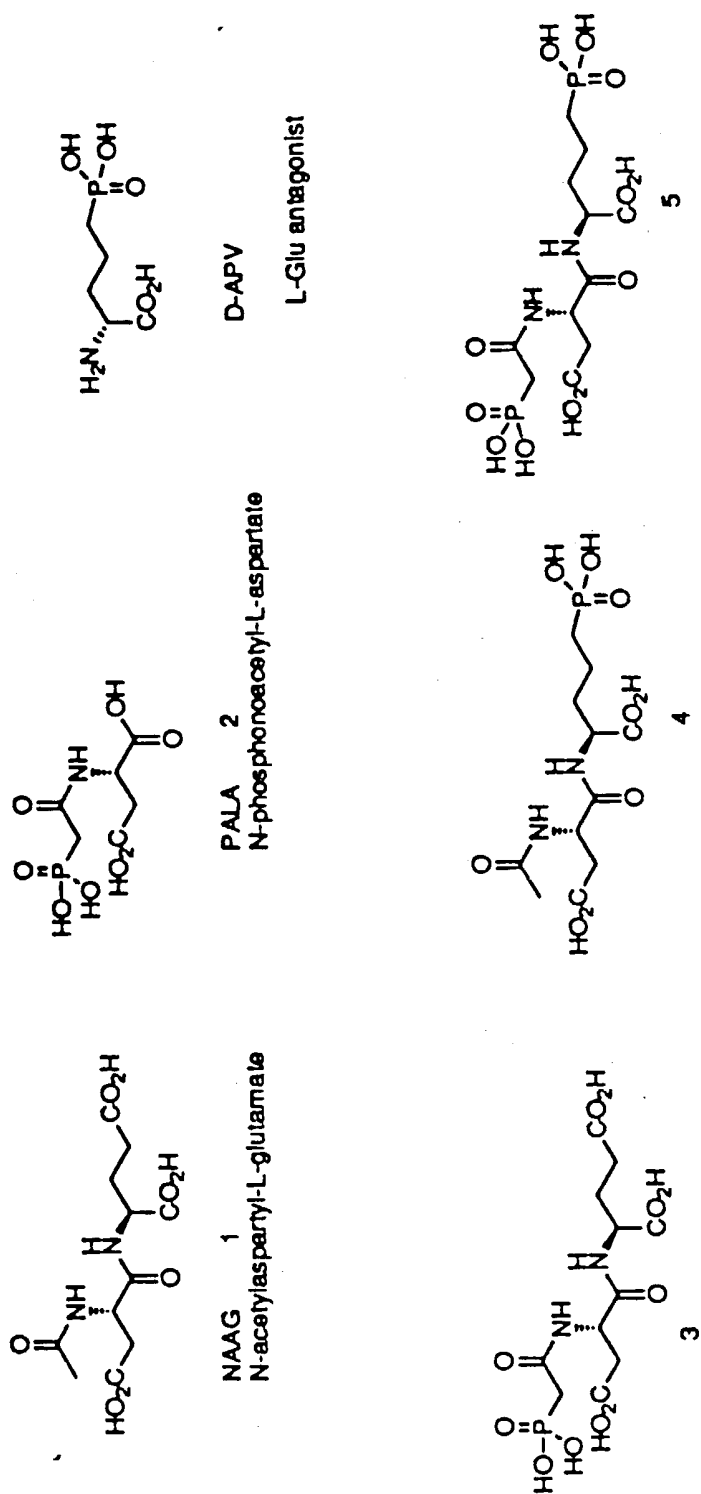
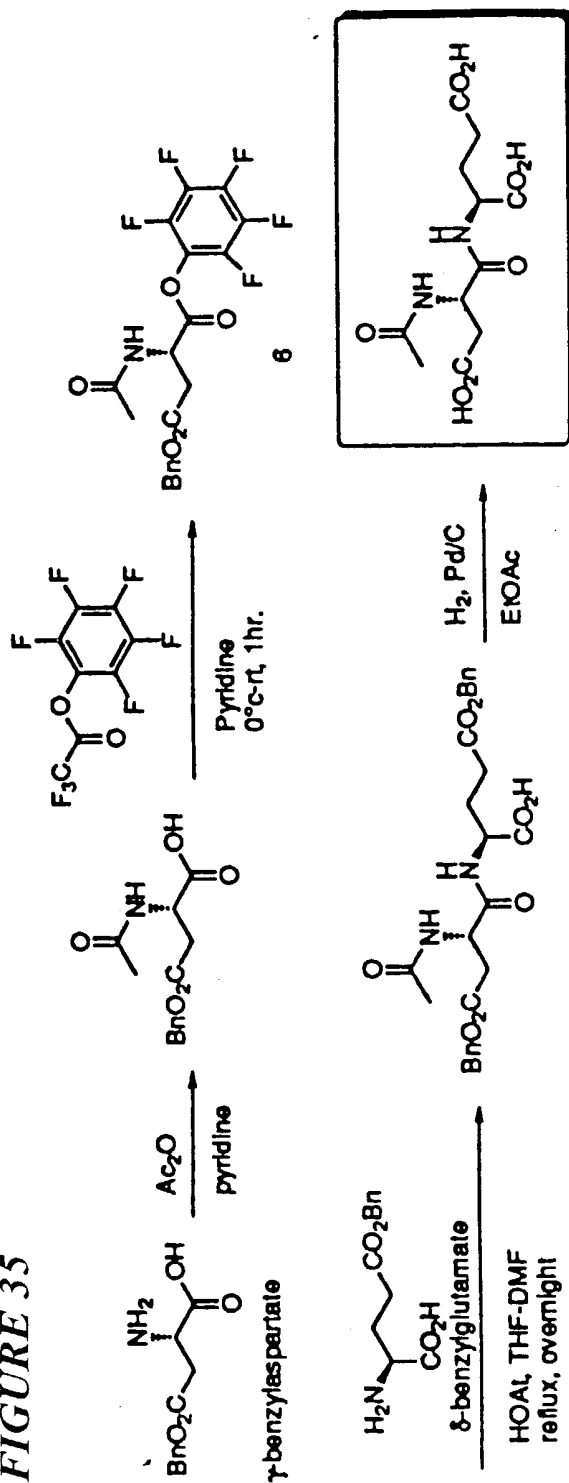


FIGURE 35



NAAG
 1
 Identical in all respects to an authentic sample from Sigma.

Ac₂O = acetic anhydride
 THF = tetrahydrofuran
 DMF = N,N-dimethylformamide
 Pd/C = palladium on charcoal
 EtOAc = ethylacetate

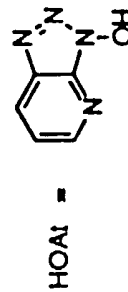


FIGURE 36

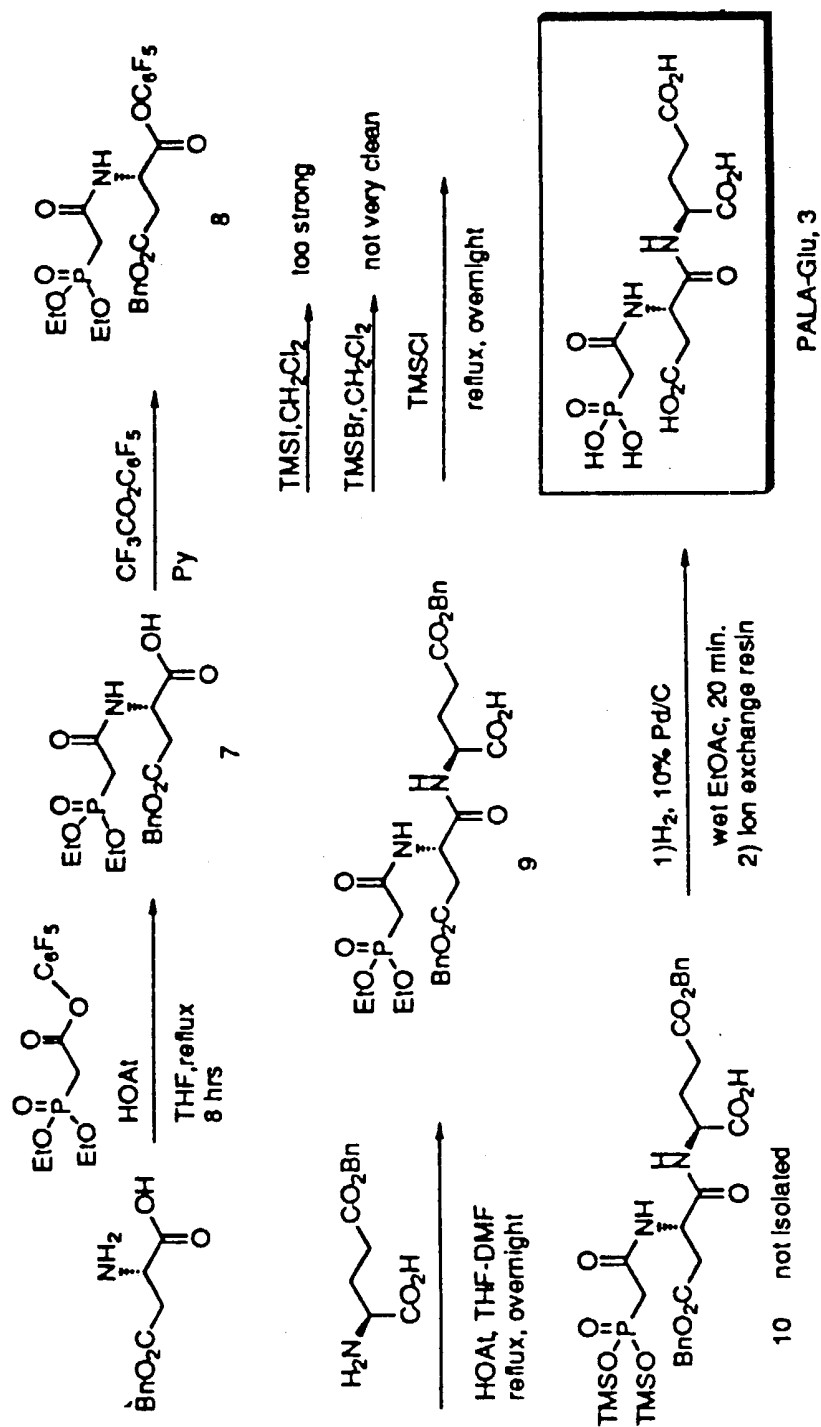


FIGURE 37

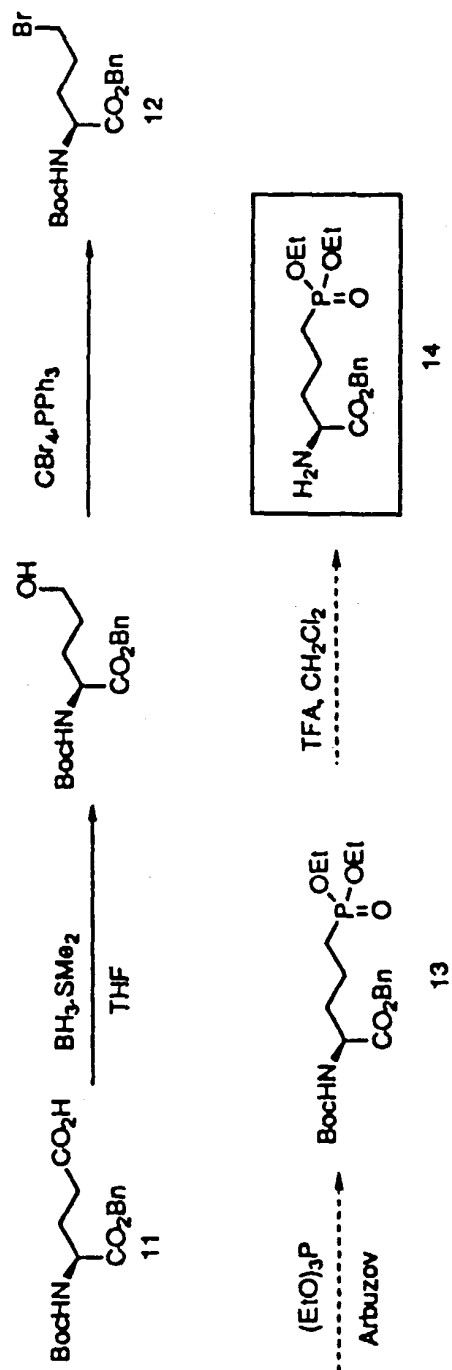


FIGURE 38

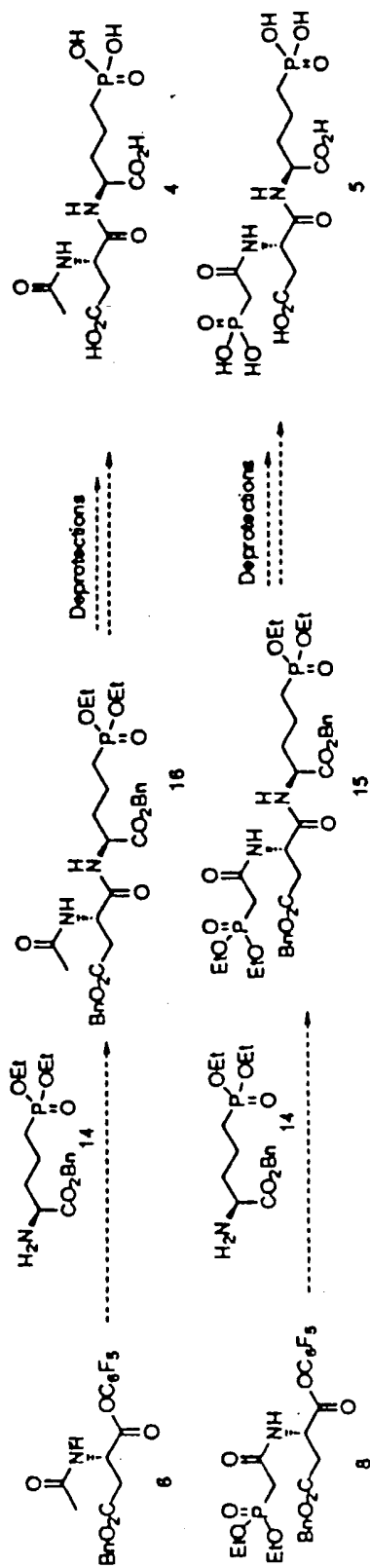
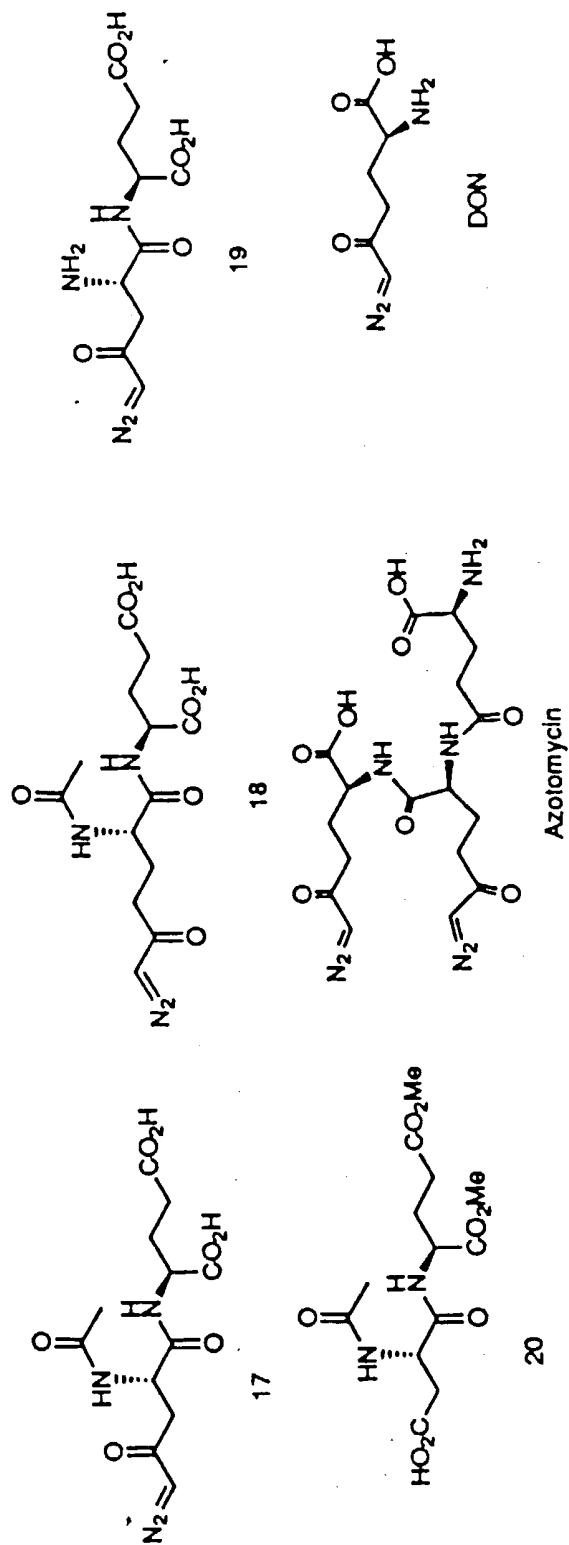


FIGURE 39



45/102

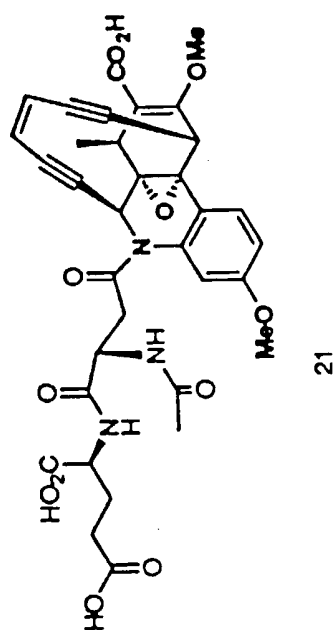
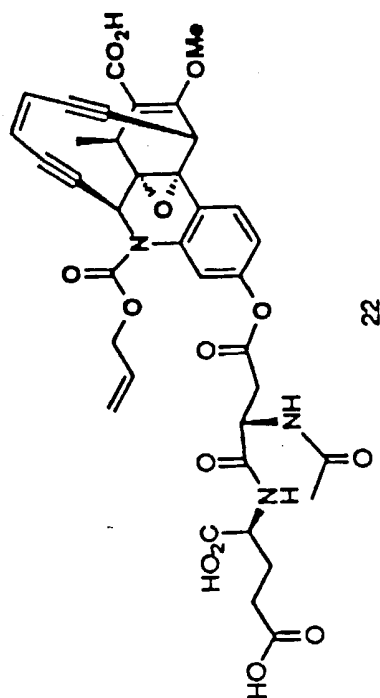


FIGURE 40

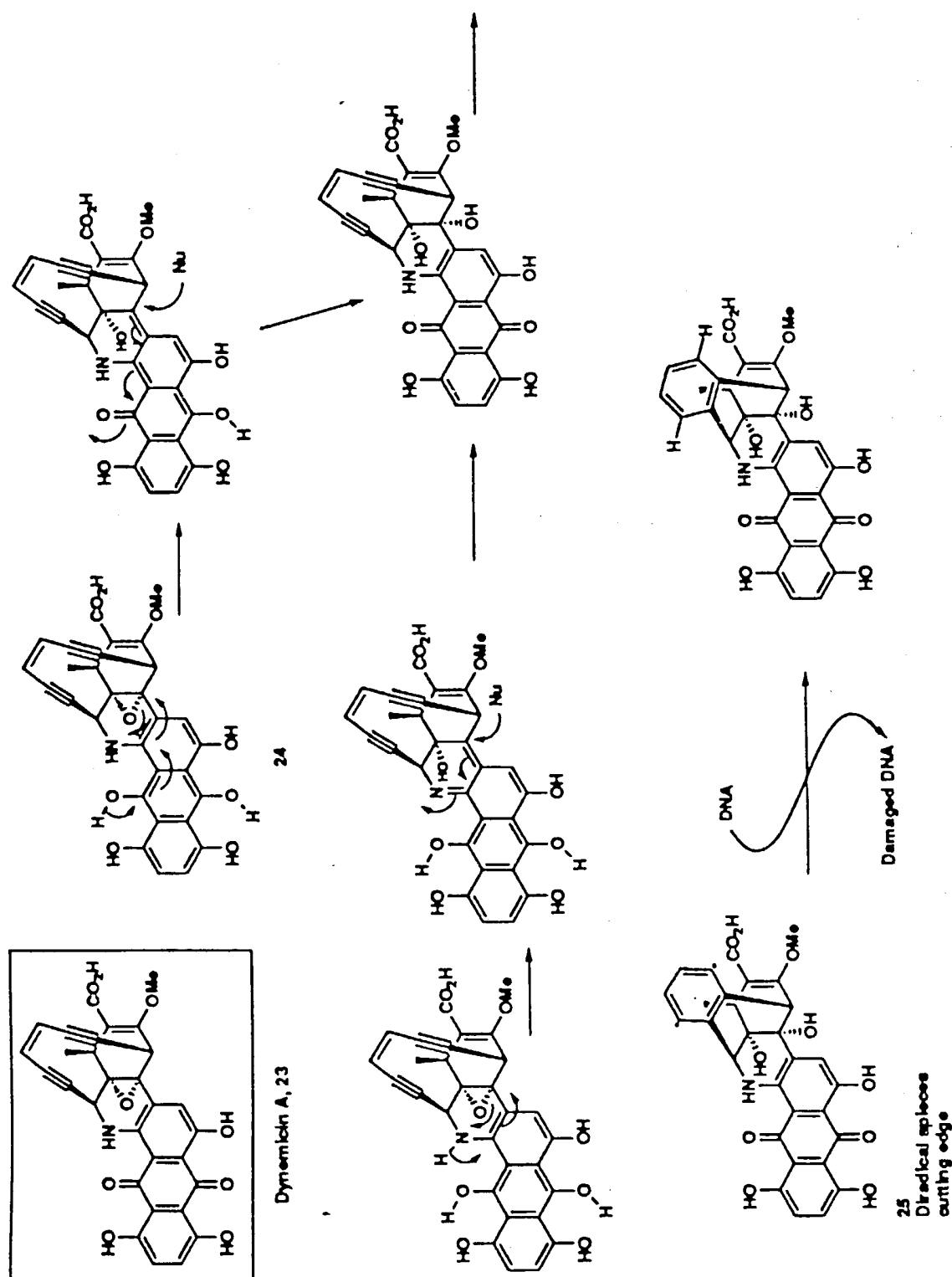
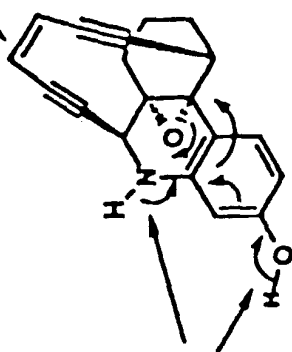
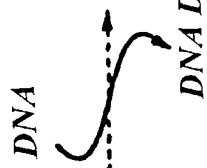
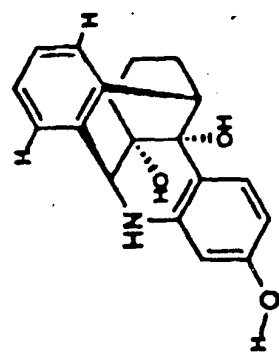


FIGURE 41

Warhead

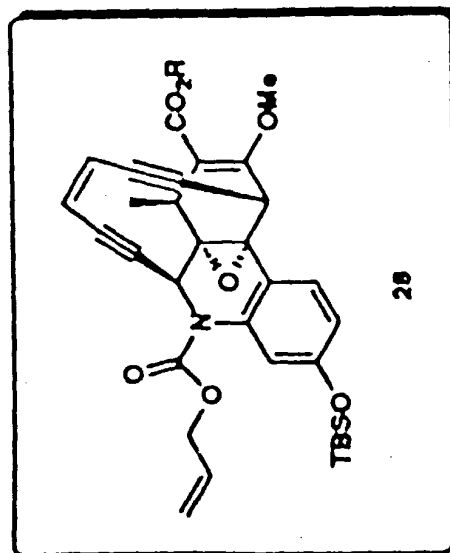
FIGURE 42



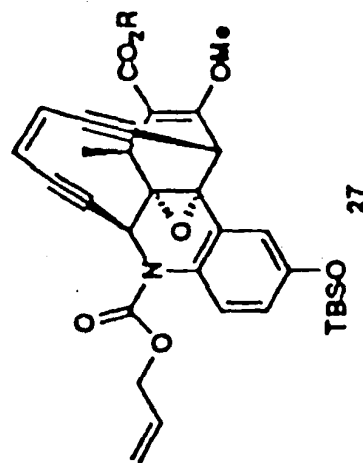
Triggering devices

26

active at the nano to picomolar levels in different cell lines readily rearranges when one or both triggering devices are deprotected



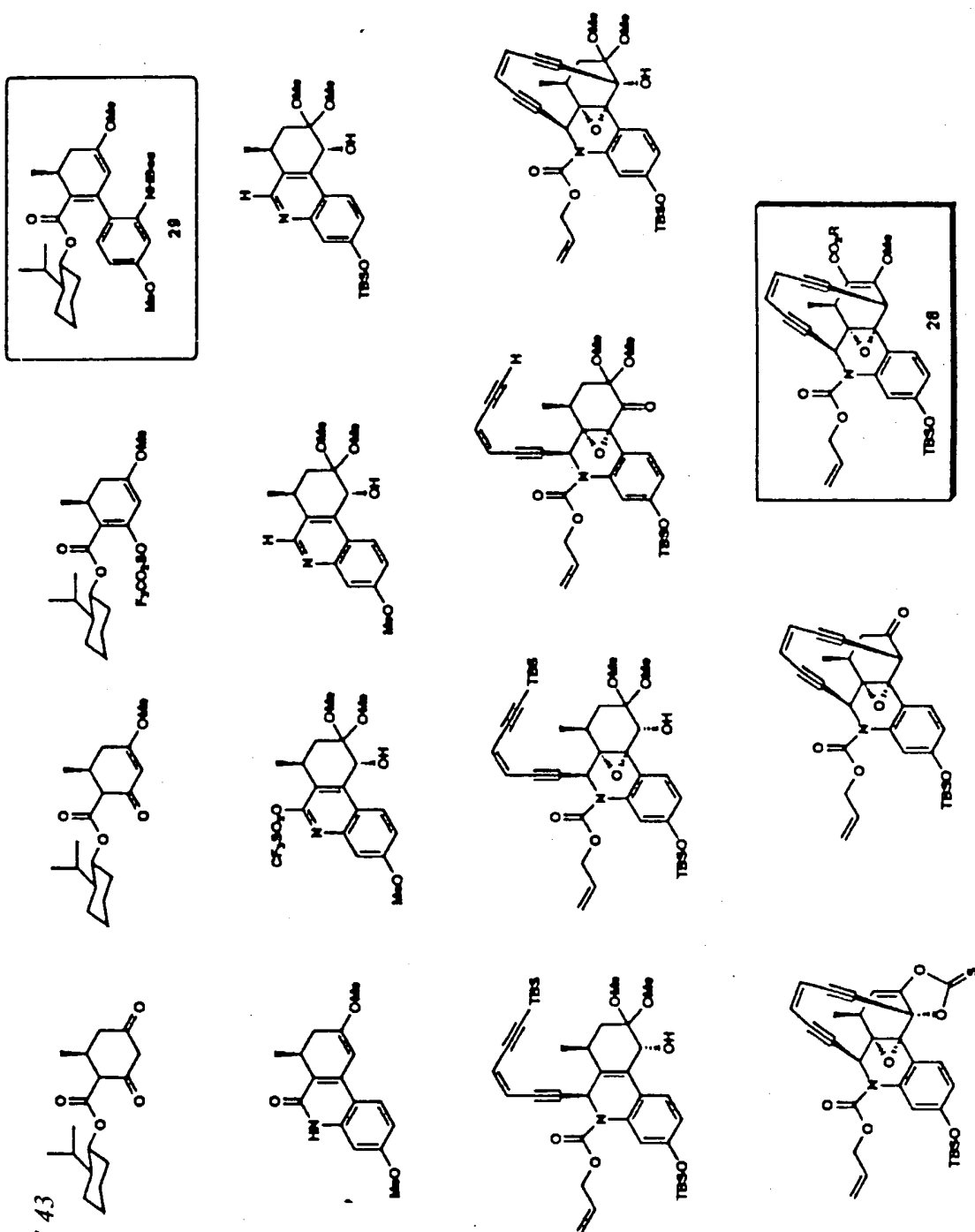
28



27

48/102

FIGURE 43



17 Steps for the optically active form 3

49/102

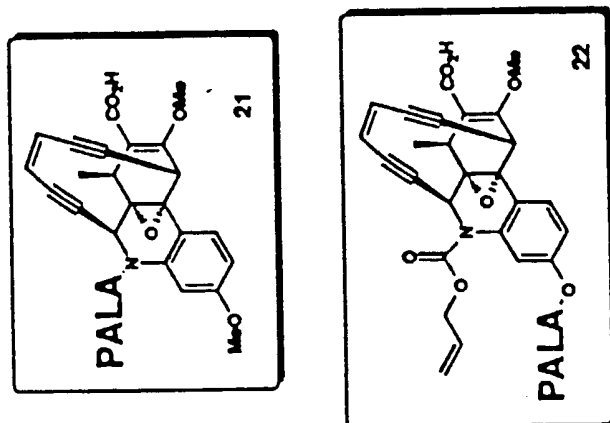
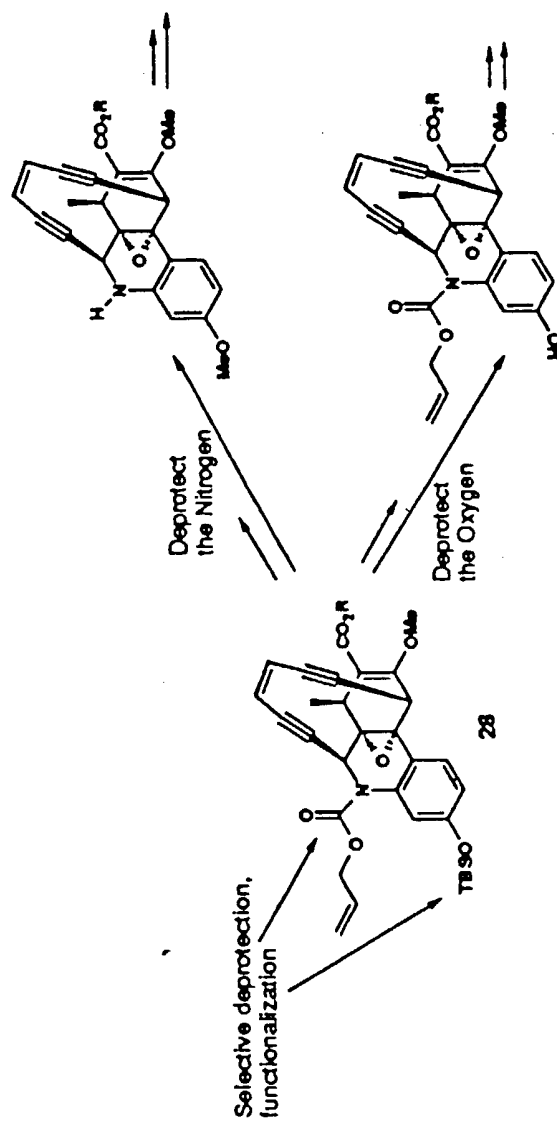


FIGURE 44



"THE GUIDANCE SYSTEM"

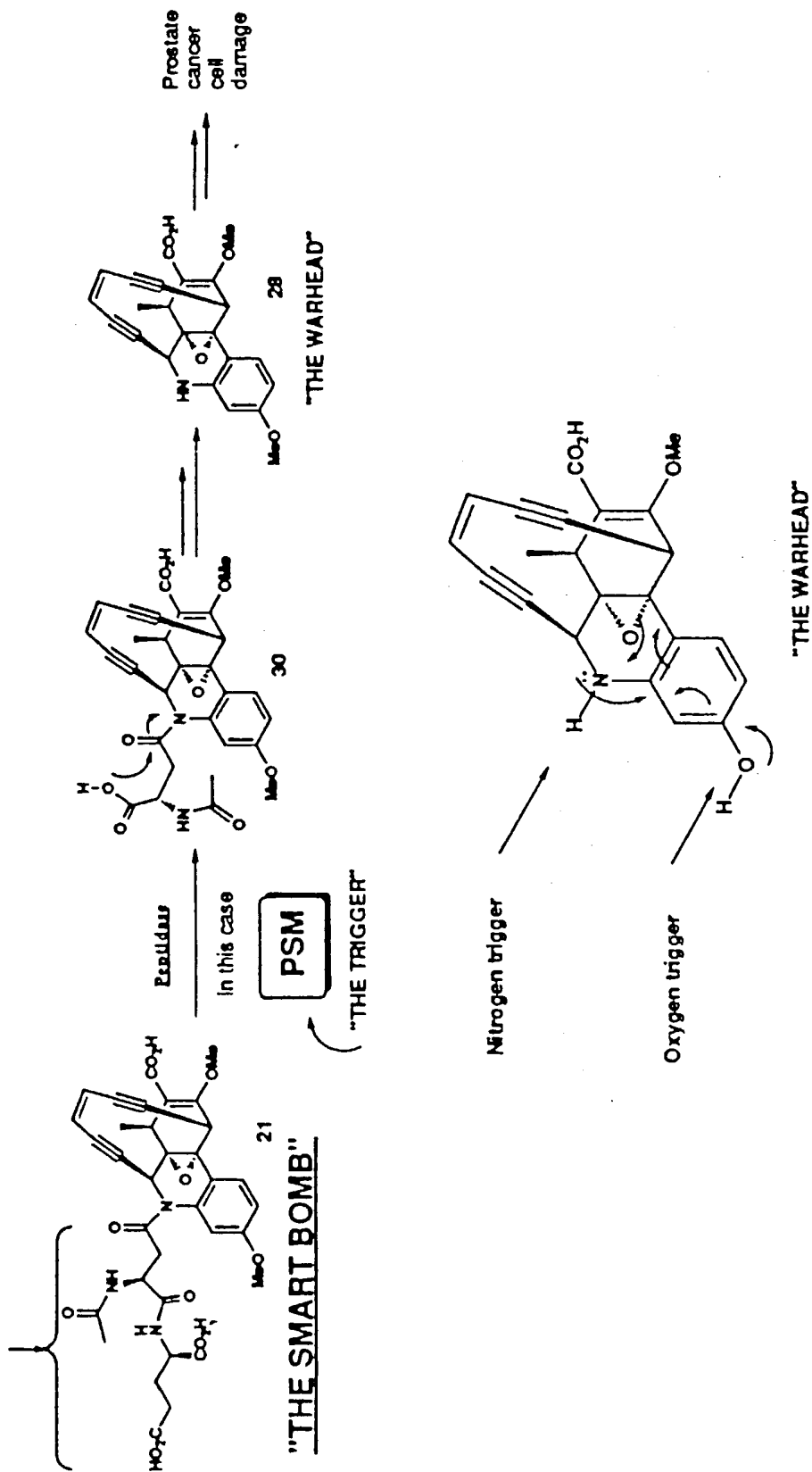


FIGURE 46A

	10	20	30	40	50	60
1	TAGGGGGGCG ATCCCCCQC	CCTGGGGAG GGAGCGCTC	AAACCTCGGA TTTGGAGCCT	GCTTCCCCG CAGAAAGGBC	TGGTGCCGC ACCACGGCGC	GTGCTGGGAC CACGACCCCTG
61	TCGGGGGTCA AGCGCCCACT	GCTGCCGAGT CGACGGCTCA	GGGATCCTGT CCCTAGGACA	TGCTGGTCTT ACGACCAGAA	CCCCAGGGGC GGGGTCCCCG	GGCGATTAGG CCGCTAATCC
121	GTGGGGGTAA CAGCCCCATT	TGTGGGGTGA ACACCCCACT	GCACCCCTCG CGTGGGAGC	AGTTAGGAGG TCAATCCTCC	AGGGTAGCTG TCCCATCGAC	GGAAACGGCTGC CCTTGCCACG
181	AGGGCTGAGT TCCCGACTCA	TCTCAGCAAG AGAGCTGTT	CTGCTGGTAG GACGACCATC	GACAGTCACT CTGTCACTGA	CAGGTTGAGG GTCCAACTCC	GTAGAACTGA CATCTTGACT
241	GAGAACCTGA CTCTTGAGCT	AACGGGGCGT TTGACCCGCA	AGGAAGGTTT TCCCTCCAA	CAAGTGCTGG GTTACGAGCC	AGCCCTGCCAA TCGGGACGTT	GACAGAGGAA CTGTCTCCTT
301	GTTTTTTTTT CAAAAAAAA	TGCTTTTGTT ACGAAACAA	TTGTTTTGTT AACAAACAA	TTGTTTTGTT AACAAACAA	TTGTTTTGTT AACAAACAA	TGTTTTGTTG ACAAACAAAC
361	TTTTTTTACC AAAAAATGG	TCTCTGTGCA AGAGACACGT	TTCTTTCTTC AAGAAAGAAG	CTTGGAAGTA GAACCTTCAT	ACAGAGGCCAA TGCTCCTCGTT	GCTTGCGAAC CGAACCCCTTG
421	TGTGTGAACC ACACACTTGG	AGGTCAGCAA TCCAGTCGTT	TCTGCACAGG AGACCTGTCC	TCTTTACCAG AGAAATGGTC	CGGTCCTTTT GCCCAGAAAA	GCTGTTTTTC CGACAAAAAG
481	CTGGGTAAGT GACCCATGAC	ATTGGCAGAC TAAAGTCTG	TTGATCCCAAC AACTAGGTTG	TTTCTAAGAA AAAGATTCTT	AAACAGAACCC TTCTGCTTGG	ACACAGGCCAA TGTGTCCTGTT
541	GCTCAGACTC CGAGTCTGAG	TTTTATTAAA AAATAAATT	TTCCAGTTTTT AAGGTCAAAA	GACTTTGCCA CTGAAACGGT	CTTCTTAGTG GAAGNATCAC	GCCTTGAAACA CGGAACCTTGT

FIGURE 46B

301 AGTTACCGAG TCCCTCTCAG CGTTAGTAC CCTATTTTAT GATGAGGATA ATATTATCTG
TCAATGGCTC AGGGAGAGTC GCAATCAATG GGATAAAATA CTACTCCTAT TATAATAGAC

561 CAATATTATG GTAATAGTAA ATAATATAGC ATGTAAATCT CCTAGCACAG TACTGGGATT
GTTAATAAC CATTATCAT TATTATATCG TACATTTAGA GGATCGTGTG ATGACCCCTAA

721 TTCGCCACTT TATTCTTCT TTTACCAAGA TACTCCTCAT TGGACTTTAA TACACAGGAC
AAGCGGTGAA ATAAAGAAGA AATGGTTCT ATGAGGAGTA ACCTGAAATT ATGTGTCTCTG

781 TAGTCTAAGG TATCACCAGG TAGTCCACTC CTGCTCGGAA TTCTTGACCC TCTTTCGGGA
ATCAGATTCC ATAGTGGTCC ATCAGGTGAG GACGAGCCTT AAGAACTGGG AGAAGGCCCT

841 TTTAGAAGAA TAGGGCATGG ACCAGATGGG TTAAACAAA TTCNAATATCT TCCACTAGCT
AAATCTTCTT ATCCCGTACC TGGTCTACCC AAATTGTGTT AAGTTATAGA AGGTGATCGA

901 TCACCTTGGG GTTGTTAAAA GATTTTIGAA CCACACACTG TGCTCATAAC AATCTTCATC
AGTGGAAACC CAACAATTTT CTAAAAACTT GGTGTGTGAC ACGAGTATTG TTAGAAGTAG

961 TCTTAAAGG ATTTATTCT TCCTGGTATT GCCCTCACTC TCATCCCTGT ATTCGGTGCT
AGAAATTTCC TAAAAAAGA AGGACCATAA CCGGAGTGG AGTAGGGACA TAAGGCCACGA

FIGURE 46C

1021 CAGTGGCTGA CACAGAAGAG TTCTTTATTG ATGTCCGCCC CCCACCCACT AGGATTCTCT
 GTCACCGACT GTGTCTTCTC AAGAAATAAC TACAGGCGGG GGGTGGSTGA TCCTAAGAGA

 1081 GCTCTCCCT CCCTTACAG GCCTCCATCC TCTTCATCCT GTTCATTTT CAGATCTCAG
 CGAGAGGGA GGGGATGTC CGGAGGTAGG AGAAGTAGGA CAAGTAAAA GTCTAGAGTC

 1141 TTCAAGCATC TCGTCCCTCAG TGTGGTGTTC CCTGATCCCT CACTCTAATC CAAGTCTTTC
 AAGTTCGTAG AGCAGGAGTC ACACCAAA GGACTAGGA GTGAGATTAG GTTCAGAAAG

 1201 TGTTTTATGC ACAGGTGGA TCTTATTTC GTTGCGTCC AATCATGTAT TTTAATATGC
 ACAAAATACG TGTCCACCTT AGAATAAGG CAAACGCAGG TTAGTACATA AATTATACG

 1261 ATGTATATAT GTATCTGCAT TTGTATGCAT GCGATTAAAG ACTAGAATAA TTAATAATTG
 TACATATATA CATACACGTA AACATACGTA CGCTAATCT TGATCTTATT AATTATTAAAC

 1321 GAAAGCTCCA TGAAAGCTGG TTGGGGACTA ATTTGTAAAC TACTTTATTC CCAGATCCTG
 CTTTCGAGGT ACTTTCGACC AACCCCTGAT TAAACATTG ATGAATAAG GGTCTAGGAC

 1381 TAAATTCTCT AAATAAACCC TGGAACTTG CCTATCTCC TTCAGGTTAA AAGCCAACTG
 ATTAAGAGA TTTATTGGG ACCTTAGAAC GGAATAGAG AAGTCCAAT TTCGGTTGAC

 1441 CAAGGTCTAA TGACTGCAGG ATCTAGCTAT CCATTGTTTC TGGCGCCTA TCGTGCACT
 GTTCCAGATT ACTGAGTCC TAGATCGATA GGTAACAAAG ACGGCGGAT ACGCACGTGA

 1501 GGGTGTCTG CAGAGAGGCT GGGTAAATG TAGTTTCATT GTAGCTGTCT GACTTGGATT
 CCCACAGACC GTCTCTCCGA CCCATTAAAC ATCAAAAGTAA CATCGACAGA CTGAACCTAA

 1561 TCTCAGCCT ACTTCACTGG AAACGCAAC TCTCACAGCA TTTTGTTTA GTTTCAGNAAT
 AGAGTGCGGA TGAAGTGACC TTTGCGTTG AGAGTGTCTG AAACAAAAAT CAAAGTCTTA

 1621 CAGAGCAAT TAGAAGTCTG AATTCTCTC AACACTTGA AATAATTAT TTATTGAAA
 GTCTCGTTA ATCTTCAGAC TTAAGGAAG TTGTGAACCT TTATTAAATA AATAAATTT

 1681 TATATTCTA ATTAATTCTG TATAAAATG TATTAAATGC TTATTGAGT CAGCAGAGGA
 ATATAAGTAT TAATTAGCA ATATTTTAC ATAATTACG AATAAACTCA GTCGTCTCCT

FIGURE 46D

1741 AGATAGAAAC TTTATGAAAG TAGAAGGTGG ATCTCCTTTT TGCCTTCATT TTCAGAACAT
TCTATCTTTG AATACTTTC ATCTTCCACC TAGAGGAAA ACGAAGTAA AAGTCTTGTA

1801 CTCGTTTACA CCCATTAGTT GAAACATTAA TGTCATTTTA TTTTCGTCCT GATTATCTCA
GAGCAAATGT GGGTAATCAA CTTTGTAATT ACAGTAAAT AAAAGCAGGA CTAATAGAGT

1861 TAAACATTT CTTAGAATAA CAGCAATACC TATCATTTGA GTTGGATAAG AAATATTTTG
ATTTTGTA AAATCTTATT GTCGTTATGG ATAGTAACTT CAACCTATTC TTTATATAAAC

1921 CAATTGGTTT GCAACTTAAA AATCTGTTTG CATGACTCTT TTTTCAGTGAA AGTAGGCAAG
GTTAAACCAA CGTTGAATTT TTAGACAAAC GTACTGAGAA AAGTCACTT TCATCCGTTT

1981 AGAAATTAAA ATTCAGAAAT ATCTCACCTA ATGTCAGAGG TAATATTGAT AATTGTGTT
TCTTTAATTT TAAGTCTTTA TAGAGTGGAT TACAGTCTCC ATTATAACTA TTAAACACAA

2041 TTACAAATAA TACATACAAC AATAATGAAA AATAAGTCCT ATCTATAGGC TCGTATCTCA
AATGTTTATT ATGTATGTTG TTATTACTTT TTATTCAGGA TAGATATCCG AGCATAGAGT

2101 TGCCTATTTT TGGATGTATT TTTC
ACGGATAAAA ACCTACATAA AAAGT

FIGURE 47A

	10	20	30	40	50	60
1	TGAAAATAC	ATCAAAAATA	GGCATGAGAT	ACGAGCCTAT	AGATAGGACT	TATTTTTTAT
	ACTTTTATG	TAGTTTTTAT	CCGTACTCTA	TGCTCGGATA	TCTATCCTGA	ATAAAAATA
61	TATTGTTOTA	TGTATTATTT	GIAAAACACA	AATTATCAAT	ATTACCTCTG	ACATTAGGTG
	ATACCAACAT	ACATAATATA	CATTTTGTTG	TTAATAGTTA	TAAATGAGAC	TGTAATCCAC
121	AGATATTCTG	AATTTTAATT	TCTCTTGCCT	ACTTTCACCTG	AAAAGAGTGC	ATGCAAAACAG
	TCTATAAGAC	TTAANAATTAA	ACAGAAACGGA	TGAAAGTGCAC	TTTTTCTCAG	TACGTTTGTC
181	ATTTTAAAGT	TGCAAAACCA	TTGCCAAAATA	TTTTTTTATC	CAACTTCAAT	GATAGGTATT
	TAAAAATTCA	ACGTTTGGTT	AACGTTTTAT	AAAAAATAG	GTTGAAGTTA	CTATCCATAA
241	GCTGTTAATT	CTAAGATATG	CATTAATTGT	TTCAACTAAT	GGGTGTCAAA	CGAGATGTTT
	CGACAATTAA	GATTCATATC	GTAATTAAAC	AAGTTGATTA	CCCACAGTTT	GCTCTACNAG
301	TGAAAATGAA	GGCAAAAAGG	AGATCCACCT	TCTACTTTCA	TAAAGTTTCT	ATCTTCCTCT
	ACTTTTACTT	CCGTTTITCC	TCTAGGTGGA	AGATGAAAGT	ATTTCAAAAG	TAGAAAGGAG
361	GCTGACTCAA	ATAAGCATTT	AATACATTTT	ATAACGAATT	AATTATGAAT	ATATTTCAAA
	CGACTGAGTT	TATTCGTAAA	TTATGTAAAA	TATTGCTTAA	TTAATACTTA	TATAAAGTTT
421	TAAATAAATT	ATTTCCAAGT	GTTGAAGGAA	ATTCAGACTT	CTAATTGCTT	CTGATTCTGA
	ATTTATTATA	TAAAGGTTC	CAACTTCCTT	TAAAGTCTGA	GATTAACGGA	GACTAAGACT

FIGURE 47B

481 AACTAAACA AATGCTCTGT GAGAGTTGC GTTCCAGTG AAGTAGCGTG AGAAATCCAA
TTGATTTTGT TTACGAGACA CTCCTCAAACG CAAAGGTCAC TTCATCGCAC TCTTTAGGTT

541 GTCAGACAGC TACATGAAC TACATTTACC AGCTCTCTGC CAGACACCAG TGCACGATAG
CAGTCTGTGG ATGTACTTTG ATGTAAATGG TCGAGAGACG GTCTGTGGTC ACGTGCTATC

601 CGCAGACAT GTAGCTAGAT CTCAGTCATA GCTNNNNNNN NNNNNNNNNN AGACCTTGCA
GCGTCTTGTA CATCGATCTA GAGTCAGTAT CGANNNNNNN NNNNNNNNNN TCTGGAACGT

661 GTGGGCTTTT AACCTGAAGG AGATAAGGCA AGATTCCAGG GTTTATTTAG AGAAATTACA
CAACCGAAA TTGGACTTCC TCTATTCCGT TCTAAGGTCC CAAATAAATC TCTTTAATGT

721 GGATCTGGGA ATAAAGTAGT TACAAATTA GTCCCCAACC AGCTTTCATG GAGCTTTCAA
CCTAGACCCCT TATTTTCATCA ATGTTTTTAAT CAGGGGTTGG TCGAAAGTAC CTCGAAAGTT

FIGURE 47C

781 TTAATTAAATTA TTCTAGTTCT TAATGGCATG CATAAATGC ACATACATAT ATACATGCAT
AATAATTAAAT AAGATCAAGA ATTAGCGTAC GTATGTTAGG TGTATGTATA TATGTACGTA

841 ATTAATAATAC ATGATTTGGAC GCAAAACGGAA ATAAGATTCC ACCTGTGCAT AAAACAGAAA
TAATTTTATG TACTAACCTG CGTTTGCCTT TATTCTAAGG TGGACACGTA TTTTGTCTTT

901 GACTTGGTTA GAGTGAAGGA TCAGGAACA CCACACTGAG GACGAGATGN NNNNNNNNN
CTGAACCAAT CTCACTCCCT AGTCCTTTGT GGTGTGACTC CTGCTCTACN NNNNNNNNN

961 NTAGTGGGTG GGGGGCGGAC ATCAATAAAG AACTCTTCTG TGTACGCCAC TGAGCACGGA
NATCACCCAC CCCCCGCGTG TAGTTATTTC TTGAGNAGAC ACAGTCGGTG ACTCGTGCCT

1021 ATAAAGGGAT GAGAGTGAGG GCNANTACCA GAAGAATAAA ATCCTTTTAA GAGATGAAGA
TATTTCCCTA CTCTCACTCC CGTNNATGGT CTCTTATTT TAGGAAAATT CTCTACTTCT

1081 TTGTTATGAG CACAGTGTGT GONTTCAAAA ATCTTTTAAAC AACCCCAAGG TOAAGCTAGT
AACAAATCTC GTGTCAACACA CCNAACTTTT TAGAAAATTG TTGGGGTTCC ACTTCGATCA

1141 TGAAGATAT TTGAATTGTG TTAACCCAT CTGGTCCTAG CCCTATTCTT TGAATCCCGA
ACCTTCTATA AACTTAAACA AATTGGGTA GACCAGGATC GGGATAAGAA ACTTAGGGCT

FIGURE 47D

1201 AAGAGGGGTCA AGAATTCCGA GCAGGAGTGG ACTACCTGGT GATACCTTAG ACTAGTCCTG
TTCTCCCAGT TCTTAAGGCT CGTCCTCACC TGATGGACCA CTATGGAATC TGATCAGGAC

1261 TGTATTAAAG TCCAATGAGG AGTATCTTGG TAAATAATA AATAAAGTCC CGAAAATCCC
ACATAAATTTC AGGTTACTCC TCATAGAACC ATTATTATAT TTATTTCAGG GCTTTTAGGG

1321 AGTACTGTGC TAGGAGATTI ACATGCTATA TTATTIAC TA TNNNNNNNNNT AATTGOCAGA
TCATGACACG ATCCTCTAA TGTACGATAT AATAAATGAT ANNNNNNNNNA TTAAACGTCT

1381 TAATATTATC CTCATCATAA AATAGGGTAA CTAAAGGCTGA GAGGGACTCG GTAACTTGT
ATTATAATAG GAGTAGTATT TTATCCCAT TATTGCGACT CTCCCTGAGC CATTGNAACAA

1441 CAAGGCCACT AAGAAAGTGGC AAAGTCAAA CTGGAATTTT AATAAAGAG TCTAGCTTGC
GTTCCGGTGA TTCTTCACCG TTTCAGTTT GACCTTAAA TTATTTTCTC AGATCGAAGC

1501 CTGTGTGGTT CTGCTTTTCT TAGAAAGTTG GANNAAGTCT CANATCAGTA CCCAGGAAA
GACACACCCAA GACGAAAAGA ATCTTICAAC CTNNTTGAGA GTHTAGTCAT GGGTCCTTTT

1561 ACAGCAAAG ACCCGCTGGT AAAGACCCTGT CCAGATMGCT GACCTGGTTC ACACANITCC

FIGURE 47E

TGTCGTTTTC TGGGCGACCA TTCTCTGGACA GGTCTAACGA CTGGACCAAG TGTGTHNAGG

1621 AAGCTTGCCT CTGTTACTTC CAAGGAAGAA AGAATGCACA GAGAGGTAAA AAAACAACA
TTCGAACGGA GACAATGAAG GTTCCTTCIT TCTTACGTGT CTCGCCATTT TTTTGTTTGT

1681 AACCAAAACAA AACAAACAA AACAAACAA AAGCAAAAAA AAACTTCCCTC
TTGGTTTGTT TTGTTTGTGTT TTGTTTGTT TTGTTTGTT TTCGTTTMTT TTTGAAGGAG

1741 TGTCCTGCAG GGCTCCAGCA CTGGAAACCT TCCTACGTCC TANTTTCAGG TTCTCTCAGT
ACAGAACGTC CCGAGGTCGT GAACCTTGA AGGATGCAGG ATNAAGTCC AAGAGAGTCA

1801 TCTACCCCTCA ACCTGAGTGA CTGTCCCTACC AGCAGCTTGT CGAGAACTCA GCCCTGCACC
AGATGGGAGT TGGACTCACT GACAGGATGG TCGTCGAACA GCTCTTGAST CGGGACGTGG

1861 GTTCCCAGCT ACCCTCCTCC TAACTCGAGG GGTGCT
CAAGGGTCGA TGGAGGAGG ATTGAGCTCC CCACGA

FIGURE 48A

1	GGATTCGCTT	10	GAGCCCTAGC	20	TCATTATGAT	30	GTCCGTGTGT	40	CCTACCCCAA	50	TAAAGACTCAT	60	ATTCTGAGTA
	CCTAAGACAA		CTCGGGATCG		AGTAATACTA		CAGGACAACA		GOATGGGTTT				
61	CCCAACTACA		TCICAATAAT		TAAAGAGAT		GGAATGAGG		TAAATAATA		ATAATATAAT		
	GGGTGATGT		ACAGTTATTA		ATTACTCTA		CCTTACTCC		ATTTTATT		TATTTATTA		
121	AAAGACAACA		TTCCCCCCCC		TTTATTATTT		TTCAATAAC		CTTCTATGAA		ATAATGTTCT		
	TTTCTTTTGT		AGGGGGGGT		AAATATAAA		AAAGTTATG		GAAGTACTT		TATTACAAGA		
181	ATCCCTCTCT		AAATATTAAT		AGAAATCAAT		ATTATTGGAA		CTGTGAATAC		CTTTAATATC		
	TAAGGAGAGA		TTTATAATTA		TCTTTAGTTA		TAAATAACCT		GACACTTATG		GAATTTATAG		
241	TCATTATCCG		GTGTCAACTA		CTTTCCTATG		ATGTTGAOTT		ACTGGGTTTA		GAAGTCGGGA		
	AGTAATAGGC		CACAGTTGAT		GAAAGCATAC		TACAACCTCA		TGACCCCAAT		CTTCAGCCCT		
301	AATAATGCTG		TAAANNNNNN		AGTTAGTCTA		CACACCAATA		TCAATATATG		TATACTTOTA		
	TTATTACGAC		ATTNNNNNNN		TCAATCAGAT		GTGTGGTTAT		AGTTTATACT		ATATGAAACAT		
361	AACCTCCAAG		CATAAAAAAA		GATACTTTAT		AAAAGAGGTT		CTTTTCTTCT		TTTTTTTTTT		
	TTGGAGGTTT		GTATTTTCT		CTATGAAATA		TTTTCTCCAA		GAATAAAGA		AAAAAANA		

FIGURE 48B

421 TCCAGATGGA GTTTCACCTCC TGTCAGGCAQ QCNQAGTGCA GTGGTGCCAT CTCGGCTCAC
AGGTCTACCT CAAAGTGAGG ACAGTCCGTC CQNTCACGT CACCACGGTA GAGCCQAGTG

481 TGCAACCTCC ACCTCCCATG TTCAAGGGAT TCTCCTTCCT CAGTCTCCTG AQTACTGGG
ACGTTGGAGG TGGAGGGTAC AAGTCCCTA AGAGGAAGGA GTCAGAGGAC TCATCGACCC

541 ATTACAGGTG TGCACCACCA CACCCAGCTA ATTTTGTAT TTTTAATAQA QACAGGGTTT
TAATGTCCAC ACGTGGTGGT GTGGGTCGAT TAAACACATA AATAATTATCT CTGTCCCAA

601 CATCGATGTT GGCCAGGCTA GTCTCGAAT CTGACCTCT AGGTGATCCA CCCGCCCTCAG
GTAGCTACAA CCGGTCCOAT CAGAAGCTTA GGAAGGAGA TCCACTAGGT QGGCCQAGTC

661 CCTCCCAAG TTGTAGAAT ACACGTGTGA QGCACTGCTC TGGCCAGGAG ATACATTTT
GGAGGGTTT ACATCTTAA TGTGCACACT CCGTGACGAG ACCGCTCCTC TATGTAAAA

721 GATAGGTTA ATTTATAAG AACTGCACA GATTGGACT TGTGGGAA TCACGATCCA
CTATCCAAAT TAAATATTTC TGTAGCTGT CTAAACCTCA ACGACCTTT AOTGCTAAGT

FIGURE 48C

781 GTATGCAATTT GACCCAGCAA TTTTATTGG TACTAATGA TTATATCTCA ATTGATCAGG
 CATACGTAAA CTGGGTGCTT AAAATTAACG ATGAATTACT AATATAGAGT TAACTAGTCC

841 TTGAACCTCG TGCAGAGAA TTGTTGTGG ACATTGAGA GGACAGTTTG GAGGCAAGGT
 AACTTGAGAC ACGCTTCITA AACACACACC TGTAACCTCT CCTGTCAAAC CTCGGTTCCA

901 ATTTTAGTAG ATTAAAGAA TTTGAATCTT GTTGGCAAGT TGGGTCATAT ACTGAGAAAG
 TAAATCAIC TAAATTCTT AAACCTTAAGA CAAACCTTCA ACCCCGATATA TGACTCTTTC

961 AGAAGACAAT GCAGATAAAT TGATATATTT ATTATGATGT ATGTTCAATA TGAAAGATCA
 TCTTCTGTTA CGTCTATTA ACTATATAAA TAATACTACA TACAAGTTAT ACTTTCTAOT

1021 CAAAATATAA CATACATNNA TCTTACTTAA CATACCTCAG TTTTAGAGGT ACCGTATGTA
 GTTTTAIAT GTATGTANNT AGAATGATTT GTATGAGIC AAAATCTGA TGGCATACAT

1081 GAAGAGTCCA TTTCTATTAA GGTAAGTTC TTTAGTCTT TTATTACTGG GCACTCTTAA
 CTTCTCAGGT AAAGATAAAT CCATTCNAGG AAATCAGGAA AATAAIGACC COTGAGAAAT

1141 TTACATGTAG CTTGAATAT GTCCAGTTG AGCAGTGAAC TGAAATGTC ATGTGATTA
 AATGTACATC GAACCTTTATA CAGGTCAAAC TCGTCACTTG ACTTTTACAG TACACTAAT

1201 GTACATATAT AATTTTTTT CATAGTAGGT CAATAACCTC CTTTATTGA CTAAATGAATC
 CATGTATATA TAAAAAATA GTATCATCCA GTTATTGGAG GAAATATACT GATTACTTAG

1261 AGTTCTCTAA TGATTATACO
 TCAAGAGATT ACTAATATGC

FIGURE 49A

	10	20	30	40	50	60
1	AATCAAAATA TTAGTTTTAT	AAACAGTTAA TTTGTCAAAT	AGTTTGATTA TCAAACATAAT	CTATAATCAA GATATTAGTT	ACACAAA AAA TGTGTTTTTT	AATGAATATT TTACTTATAA
61	ATCTTTTATG TAGAAAATAC	TCAGTAGAGG AGTCATCTCC	GTGAATGAAT CACTTACTTA	CCTTCAGGAT GGAAGTCCTA	TTTGATGATA AAACTACTAT	GTATCAGATA CATAGTCTAT
121	CCCAGCACTA GGGTCGTGAT	TGCTAGAAAT ACGATCTTCA	TGTGAAGAAT ACACITCTTA	TCACGAGATG AGTGCTCTAC	AATAAATCAC TTATTTAGTG	AGATTCTGTC TCTAAGACAG
181	CTCAAAATGG GAGTTTACC	TTAGATCTAT AATCTAGATA	TCAGGAAACA AGTCCTTTGT	AAGCTRAAAA TTCGATTTTT	AACCCACCCA TTGGGGTGGT	ATAACTAANA TATTGATTTT
241	ATCAACCMAA TAGTTGGTTT	TGAANAACAA ACTTTTGTGT	CAATCATAAA GTTAGTATTT	ATAAGTAAGT TATTCATTTA	ACCTATAGAA TGGATAATCTT	AGAAAAGCTC TCTTTTCGAG
301	AGAGGAGGTA TCTCCTCCAT	AAAAGATAAC TTTTCTATTG	TCTTCCAAAA AGAAGGTTTT	GGAATACTAT CCTTATGATA	ATACTGTA AA TATGACATTT	CTGTGTACTG GACACATGAC
361	ATAGAAGGAA TATCTTCCTT	GAATTAGAAA CTTAATCTTT	NNNNNNNNTG NNNNNNNNAC	TAAGTGGCAT ATTCACCGTA	ACATACTAAG TGTATGATTC	CTAAGTGTAA GATCACACTT

FIGURE 49B

421 CACAAGCCTA AATATGTAGT TGCTTCACAG AAGTTAGAA GTAAATTAAAC CTCATGAATT
GTGTTCCGGAT TTATACATCA ACGAAGTGTC TTCCAATCTT CATTTAATTG GAGTACTTAA

481 TCTTGAGAGA ACTTGTAAGG ACTAAGCTTT CGATTITGGA GAAAGATTTT AATACCAAAAT
AGAACTCTCT TGAACATTCC TGATTGAAA GCIAAAACCT CTTTCTAATAA TTAIGGTTTA

541 AAAAAGTACC TTTGTTTGGT AATCTCAATC ATTATAATAG TGCTTAGATA ATACCTAGGA
TTTTTTCATGG AAACAAAACCA TTAGAGTTAG TAAATATTATC ACGAATCTAT TATGGATCCT

601 ACAAAATTAA TATTAAATTT ACTTTAAAAA AAAGTACAIG ATIGGGGAAT CACAACTGGC
TGTTTAATIT ATAATTTAAA TGAATTTTTT TTTCATGTAC TAACCCCTTA GTGTTGACCG

661 CTTACTAGAT TCTCTNNNNN NATATGCACT GAAAGAATG AAAAAACACTG AACCATAATAT
GAATGATCTA AGAGANNNNN NTATACGTGA CTTTICTTAC TTTTGTGTGAC TTGGTTTATA

721 NTGTTTTTTT AAGTTTAAAA TTAATTGGA AAAAATAGT AAGGAATATC AGAAGCAAAA
NACAAAAAAA TTCAAATTTT AATTAAACCT TTTTATTATCA TTCCCTTATAG TCTTCGTTTT

FIGURE 49C

```

781 AAATAAAATG AAAGCAAGAA TCCTCAGAGG TAGCACCAGAA TTTGGGCTTTG CTTAGATGGA
   TTTATTTTAC TTTGCTTCTT AGGAGTCTCC ATCGTGCTTT AAACCGAAAC GAAATCTACCT

841 TCTATCAAAG CTATGGCCCA TGAAGAAGAT TCAGGAGTTA GTTTAAAGCT GGTTCACATA
   AGATAGTTTC GATACCGGGT ACTTTTCCTA AGTCCTCAAT CAAATTTCGA CCAAGTGTAT

901 ATGGAATCTA GCAGAAGACT GTGCATAAAG GTGOTCTAAG AACAAACAATA TCCTGACCAG
   TACCTTAGAT CGTCTTCTGA CACGTATTTC CACCAGATTTC TTGTTGTTAT AGGACTGGTC

961 GTGAGGGGGC TCACNCTNAA TNCCAGCACT TTGGGAGCCC AAGGTGGGTG GATCACCAAG
   CACTCCCCCG AGTGNGANTT ANGGTCGIGA AACCTCGGG TTCCACCCAC CTAGTGCTCC

1021 TCAGGAGTTT GAGACCAGCC TGACCAACAT GGTGAACCCG CGTCTCTACT AAAAATAGAA
   AGTCCTCAAA CTCTGGTCGG ACTGGTTGTA CCACTTTGGC GCAGAGATGA TTTTATCTTT

1081 AAATTAGCCG NGCCTACGTG CTTCTAATCC CAGCTGAAC T CAGGAGACTG AGACAAGAGA
   TTTAATCGGC NCGGATGCAC GAAGATTAGG GTCGACTTGA GTCCTCTGAC TCTGTCCTCT

1141 ATCACTTGAA CCCAGCATGC AAGCTTNNNN NNGCCACTGC ACTCCAGCCT AGGDTGCAAA
   TAGTGAACTT GGGTCGTACG TTCGAANNNN NNCGGTGACG TGAGGTGCGA TCCCACGTTT

1201 AAAAAAAAAA ANGACACATT ACTCAGGTAA GGTAATCAAT AA
   TTTTTTTTTT TNCGTGTAA TGAGTCCATT CCATTAGTA TT

```

FIGURE 50A

- AAGGTA AAAAATTATCTCTTTTCTCTCCCCCAATGTA AAAAAGTTATAG -
- AAGGTA AAAAATTATCTCTTTTCTCTCCCCCAATGTA AAAAAGTTATAG -
- TGGGTTTTACATGTGTAGAAATCATTTTCTTAAAACTTTATGAATACCATT -
- TGGGTTTTACATGTGTAGAAATCATTTTCTTAAAACTTTATGAATACCATT -
- ATTTTCTTGTAATCTGTGACATGCCACCTTACAGAGAGGACACATTTAC -
- ATTTTCTTGTAATCTGTGACATGCCACCTTACAGAGAGGACACATTTAC -
- TAGGTTATATCCCGGGGTTAAATTCGAGCATTGGAATTTGGCCAGTGCTAG -
- TAGGTTATATCCCGGGGTTAAATTCGAGCATTGGAATTTGGCCAGTGCTAG -
- ATGTTTAGAGTGAAACAGAA CAAATTTTCTGTGCTTACAGGTTATGGCTG -
- ATGTTTAGAGTGAAACAGAA CAAATTTTCTGTGCTTACAGGTTATGGCTG -
- TGGCCTACAAGAAGCATGCACTGGGTTTATTATTAACTTTCAGTATCTTT -
- TGGCCTACAAGAAGCATGCACTGGGTTTATTATTAACTTTCAGTATCTTT -
- GTTTTAAATATTTTCTACAAAAATGTTTACTAAATTAAATTGTAGTATGA -
- GTTTTAAATATTTTCTACAAAAATGTTTACTAAATTAAATTGTAGTATGA -
- ATTGTTATAAATAATGAGGGAAAA CAATTTACACATAGCAAATTTAAAAA -
- ATTGTTATAAATAATGAGGGAAAA CAATTTACACATAGCAAATTTAAAAA -
- TTAGTGTCATTTGATTTGTTAATATATTTTCTCTTTAGTGGGAAATTAA -
- TTAGTGTCATTTGATTTGTTAATATATTTTCTCTTTAGTGGGAAATTAA -
- ATTTTAAAAAATTCCTTTTCGACTGTAGAACAAATAGGAATTTGGCCTGT

FIGURE 50B

|||||
- ATTTTAAATAATTCCTTTTCGACTGTAGAACAATAGGAATTTGGCCTGT -
- GGGGTCTACTTGCTTATTATATTGTAAAGCTAGTGGTAGGAATAGCAAA -
- GGGGTCTACTTGCTTATTATATTGTAAAGCTAGTGGTAGGAATAGCAAA -
- TGCTCACTACCACTAATAAGAACATTTTCTAAATCTGATGTTCTGAGGATT -
- TGCTCACTACCACTAATAAGAACATTTTCTAAATCTGATGTTCTGAGGATT -
- TTTAGAGCCTTATAGTAGCAAAAGAAAGGGAAATTCTATCCGAGATGTC -
- TTTAGAGCCTTATAGTAGCAAAAGAAAGGGAAATTCTATCCGAGATGTC -
- CTTTGTGTAGGCCCTAATGAGAAAGGTTGAAGATAAAGTTCTGGTACTC -
- CTTTGTGTAGGCCCTAATGAGAAAGGTTGAAGATAAAGTTCTGGTACTC -
- ATTTAAGTGTAATATTGAAATTTGATATTACCGAATCTGGAAACAACCAAT -
- ATTTAAGTGTAATATTGAAATTTGATATTACCGAATCTGGAAACAACCAAT -
- TTAAATAAGGAAGAAGACACACTGTGTTTCT -
- TTAAATAAGGAAGAAGACACACTGTGTTTCT -

FIGURE 51A

	10	20	30	40	50	60
1	AGAAACACA TCTTTTGTGT	GTGTCCTTCT CACAGAAAGA	TTCCCTTATTT AAGGAATAAA	TAAATTGGTT ATTAAACCAA	GTCCAGATT CAAGGTCTAA	CGGTAATATC GCCATTATAG
61	AATTTCAAT TTAAAGTTA	ATTACACTTA TAATGTGAAT	AATGAGTACC TTACTCAITGG	AGAACTTTAT TCTIGAAATA	CTTCAACCTT GAAGTTGGA	TTCTCATTAG AAGAOTAATC
121	GCCTACAACA CGGATGTTGT	AAOGACATCT TTCCTGTAGA	CGGATAGAAT GCCTATCTTA	TTCCCTTTTC AAGGGAAG	TTTTTGCTAC AAAAACGATG	TATAAGCTCT ATATTCCGAGA
181	AAAAATCCTC TTTTTAGGAG	AGACATCAG TCTTGCTAGIC	ATTIAGAAAT TAAATCTTTA	GTCTTATTA CAAGAATAAT	GTGGTAGTGA CACCATCACT	GCATTGTGCTA CGTAAACGAT
241	TTTCCTACCA AAAGGATGGT	CTAGCTTACA GATCGAATGT	AATATAATAA TTATATTATT	GCAAGTAGAC CGTTCATCTG	CCCACAGGCC GGGTGTCCGG	AAATTCCCTAT TTTAAGGATA
301	TTGTTCTACA AACCAAGATGT	GTCGAAAGGG CAGCTTTCCC	AATTTTAA TTAAAAAATT	AATTAAATTT TTAAATTA	CCCACATAAG GGGTGATITC	AGAAAATAT TCTTTTATA
361	ATTAACAAAT TAATTGTTTA	CAATGACAG GTTTACTGTC	TAATTTTAA ATTAAAAAATT	ATTGCTATG TAAACGATAC	TGTAATTTGT ACATTTAACA	TTTCCCTCAT AAAGGGAGTA
421	TATTTATAAC ATAAATATTG	AATTCATACT TTAAGTATGA	ACAAATTTAAT TGTTAAATTA	TTAGTAACA AATCATTTGT	TTTTTGTA AAAAACATCT	AAATATTAA TTTATNAATT

FIGURE 51B

481 AACAAAGATA CTGAAGTTA ATATNAAACC CAGTGCATGC TTCTTGTAGG CCACAGCCAT
TTGTTTCTAT GACTTTCAAT TATANTTTGG GTCACGTACG AAGAACATCC GGTGTCGGTA

541 AACCTGTAAG CACAGAAAAA TTGTCTCTGT TACTCTAAAC ATCTACACTG GCCAAATTCC
TTGOACATTC GTGTCTTTT AAACAAGACA AIGAGATTG TAGATGTGAC CGGTTTAAGG

601 AATGCTCGAA TTTAACCCCG GGATATAACC TAGTAAATGT GTCCTCTCTG TAAGGTGGCG
TTACGAGCTT AATTGGGGC CCTATATTGG ATCATTTACA CAGGAGAGAC ATCCACCCG

661 ATGTCACAGA ATACAAGAA ATAATGGTAT TCATAAACTT TTAAGAAAT GATTCTACAC
TACAGTGTCT TATGTTCTTT TATTACCATA AGTATTTCAA AATTCTTTA CTAAGATGTG

721 ATGTAAACC CACTATAACT TTTTACATTG GGGGAGAGAA AAAAGAGAT AATTTTACC
TACATTTTGG GTGATATTGA AAAATGIAAC CCCCTCTCTT TTTTCTCTA TTAANAATGG

781 TT
AA

FIGURE 52A

1	GATGCTATTT	10	GGGCAATTC	20	TTATTGACAG	30	TTTTGAAATG	40	TTAGGCTTTT	50	ATCTCCATTT
	CTACGATAAA		CCC GTTAAAG		AATAACTGTC		AAACITTTAC		ATCCGAAAA		TAGAGGTTAA
61	TTTAGTACIT		AAATTTTCCA		ACATGGGTGT		TGCTTGTTAT		TTTATCAGTA		TAAATAGAA
	AAATCATGAA		TTTAAAGGT		TGTACCCACA		ACGAACAATA		AAATAGTCAT		ATTTTATCTT
121	GAGTGGTTCT		GTTCTGGAAT		TTAGTATATA		CATGAGTATC		TAGTGTATGT		CAGCCATGAA
	CTCACCAAGA		CAAGACCTTA		AATCATATAT		GIACATCATAG		ATCACATACA		GTCGGTACTT
181	AATGAACCTT		TCAGATGTTT		AACCTCAGGG		AACCTAATIG		AGTCATIGCT		CCAGACATTG
	TTACTTGGAA		AGCTACAAA		TTGAGTCCCC		TTGGATTAAAC		TCAGTAAACGA		GGTCTGTAAC
241	TTGCTTTGAA		CCCACTATAT		TNNNNNNNCT		CGGGCAATGA		CTCAGTGTGG		CAAGGATACT
	AACGAAACTT		GGGTGATATA		ANNNNNNNGA		GCCCCGTTACT		GAGTCACACC		GTTCCCTATGA
301	ACTGCAGGCC		TGTTTCTGGA		AGGCACIGGA		CTCCTCTGAT		GCAAACTTTG		CCCAGGGACT
	TGACGTCCGG		ACAAAGACCT		TCCGTGACCT		GAGGAGACTA		CGTTTGAAC		CGGTCCCTGA
361	CCTTGATAGC		TCTTAAATAG		ATGCTGCACC		AACACICTCT		TTCTTTTCTC		TCTTTTCTT
	GGAACTATCG		AGAAATTATC		TACGACGTGG		TTGTGAGAGA		AAGAAAGAG		AGAAAGAGAA

FIGURE 52B

421 TATTCAATAT TAGACTACAA GCAGTCTAAG GACTTCTCAG GGTTCCTAGC TCTCTCTCAT
ATAAGTTATA ATCTGATGTT CGTCAGATTG CTGAGAGATC CCAAGATCG AGAGAGAGTA

481 TTCACACATG CTTTCCTAGT AATCTCTACT CAIATATCTT ACTOCTACGC TGGGGCCAGA
AAGTGTGTAC GAAAGGATCA TTAGAGATGA GTATATAGAA TGACGATGCG ACCCCGGTCT

541 TAACNNNNNN CTTCATTCTT GTTTTATCT CTATTCITCT TCCCCTTCTG CTTTCATTAT
ATTGNNNNNN GAAGTAAAA CAAAAATAGA GATAAGAAGA AGGGGAAGAC GAAAGTAATA

601 TGAACACTTC TGCTTTCATT ATTGAAACTT TCCCAGATTG GTTCTGCTTA ACCTGGCATT
ACTTTGAAAG ACGAAAGTAA TAACTTTGAA AGGCTCTAAA CAAGACGAAT TGGACCGTAA

661 GGAAC TGTTT CCTCTTCCCT GTGCTGCTTT CTCCCATTGC CATGTCCTTT TTTTTTTTTT
CCTTGACAAA GGAGAAGGGA CACGACGAAA GAGGGTAACG GTACAGGAAA AAAAAA

721 TTTTTTTTTT TGAACAGTG TCACTCTGTT GCCCAGGCTG GAGTGCAATG GTGCAATCTT
AAAAA AAAA ACTCTGTCTAC AGTGAGACAA CGGGTCCGAC CTCACGTTAC CACGTTAGAA

FIGURE 52C

781 GCCCACTGCA ACCCCGCCCT CCGGGTTC A GTGATTCTC CTGCCTCAGC CTCCTGAGTA
CCGGTGACGT TGGGGGCGGA GGCCCCAGT TCACTAAGAG GACGGAGTCG GAGGACTCAT

841 GCTGGGATTA CAGGTGCCCCA CCACTATGCC CGGCTGATTT TTGTATTTTT AGTAGAGATN
CGACCCTAAT GTCCACGGGT GTGATACGG GCCGACTAAA AACATAAAAA TCATCTCTAN

901 NNNNNNTTT CACCATNGCT GATCAGGCTG GTCTCGAACT CCTGACCGCA GTGANTCCGC
NNNNNNNAAA GTGGTANCGA CTAGTCCGAC CAGAGCTTGA GGA CTGGCGT CACTNAGGCG

961 CCTCCTTGGC CTCCCAAGT GCTGACATTA CAGGCATQAG TCACTGCCNC CAGCCACCAT
GGAGGAACCG GAGGGTTTCA CGACTCTAAT GTCCGTACTC AGTGACGCG GTCCGGTGGA

1021 TATTCTCTAG AGGTGAGAGA ACACTGGCTC TTCTAACAA TTGAAATTTG ATAGAGACC
ATAAGAGATC TCCACTCTCT TGTGACCGAG AAGATTGTTC AACTTTAAAC TATCTCTGO

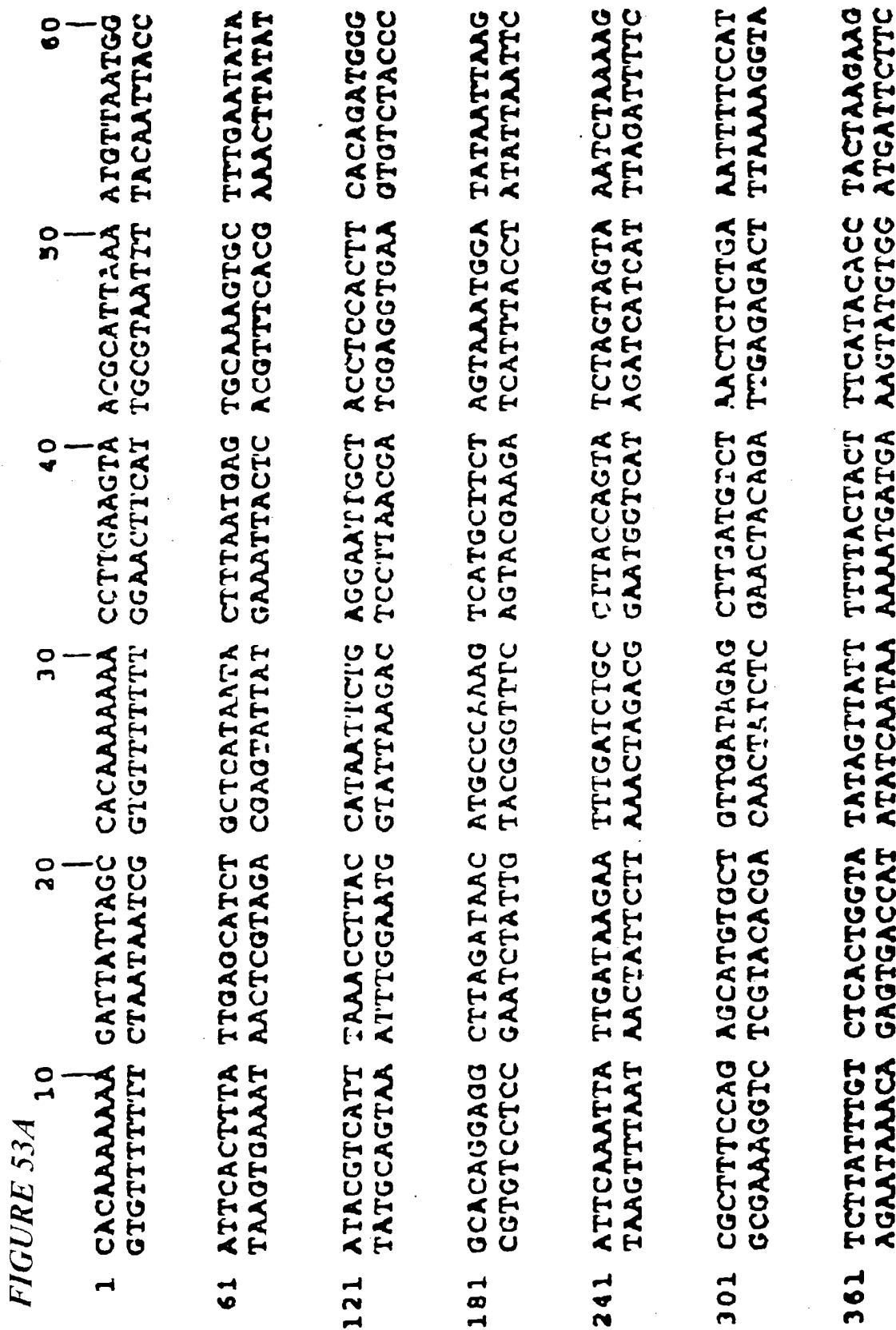


FIGURE 53B

421 ACAGGAGGAT CAAAGATAGG ATTTCAATTA GAATGCCCTAA AGCTTCACGT ATTTAATTC
TGTCCTCCTA GTTCTATCC TAAAGTAAAT CTTACGGATT TCGAAGTGCA TAAATTTAAG

481 AGAATAAGAT TCAGGCAGAC CACCAGTATA TGCCATGGTC CCTGGTTATC TTTCAGCAGG
TCTTATTCTA AGICCGTCTG GGGTCATAT ACGGTACCAG GACCAATAG AAAGTCGTCC

541 TGACCGAGAA AGAAACATG GTATGTITA TGAATCGTG GGTCTTGTA GTTCACTTC
ACTGGCTCTT TCTTTGTAC CATTACAAAT ACTTIACCAC CCAAGNACAT CAAAGTGAAG

601 AACATATCTG CCTTTACTGT ATTAAGATGA TGGATTAACT TATTCTGTAT ATGGGCATGT
TTGTATAGAC GGAATGACA TAATICTACT ACCTAATTGA ATAAGAACTA TACCCGTACA

661 AAACAATAT ACTTTTACTA AACAGCTACA GAGAGACAAA TGTGTTTCCA GACAACTTA
TTTTGTATA TGAAAATGAT TTGTCGATGT CTCCTGTGTT ACACAAAGGT CTGTTTGAAT

721 AGAGACTGAG TGTTCNAACT GAATAATCTC GACCTTAATT GTAACATAT TTTATGAAAT
TCTCTGACTC ACAAGTTTGA CTTATTAGAG CTGGAATTAA CATTGATATA AAATACTTTA

FIGURE 53C

781	CCAGCTGTAA	GGCAAAAACA	GACTTCTTTG	GGCTACCAC	GGGCATTTTG	TTCCTGTTAN
	GGTCGACATT	CCGTTTTTGT	CTGAAGAAAC	CCGGATGGTG	CCCGTAAAC	AAGGACAATN
841	NNNACTCCA	AACCTTAAAC	CCACGTCCAC	TTAAATRAAG	GCCTGGAAAT	AAATGTCATT
	NNNATGAGGT	TTGGAATTG	GGTGCAGGTG	AATTTATTAC	CGGACCTTTA	TTTACAQTAA
901	ATCTGATATT	ATACTGAGAT	GTTTAGTTAT	GAAATCAAAA	GTGGAGAATT	TCAATCTGTC
	TAGACTATAA	TATGACTCTA	CAAAATCAATA	CTTTAGTTTT	CACCTCTTAA	AGTTAGACAG
961	CTGTAAGCTT	TCTCTGCGGT	CACGACCCTC	ATGCACTCAG	GCTGTGCGGT	GCAGCATGCT
	GACATTGCGAA	AGAGACGCCA	GTGCTGGGAG	TACGTGAGTC	CGACACGCCA	CGTCGTACCA
1021	CTGTCAATGTC	TGTTTTTCTTC	TGCCCTGTACA	CGGGTGGTTG	TTCCCTGTCTA	CCTGTTTGAG
	GACAGTACAG	ACAAAAGAAG	ACGGACATGT	GCCCCACCAAC	AAGGACAGAT	GGACAAACTC
1081	GAAATATGAA	TACGTNNNNN	NCTAGAAATCT	ACTGCACATG	CAATAAGGAA	ACAATCAGTA
	CTTTATACTT	ATGCANNNNN	NGATCTTAGA	TGACGTGTAC	GTTATTCCTT	TGTTAGTCAT
1141	AGAAATCACTT	TCTCGTGGAA	AATTCATTAG	AATTAACATC	TCGTTTTTAA	ATGCTCTATC
	TCTTAGTGAA	AGAGCACCTT	TTAAGTAATC	TTAATTGTAG	AGCAAATTT	TACGAGATAG

FIGURE 53D

1201 AAGGTGTAAG TAATTCCTCT CTCTTTTCCC TTITTCACTA AGGAGTTTGT ATATTAAACA
TTTCACATTT ATTAAGGAGA GAGAAAAGGG AAAAAGTGAT TCCTCAAACA TATAATTGT

1261 GAATTTCAG TAATGTATTA TAAATTTATT TAANNTATT ACAATAAAAT GCCACGTATA
CTTAAGGTTT ATTACATAAT ATTAATAA ATNNATAAA TOTTATTTA CCGTGCATAT

1321 AGCATCAAGC AACATGANN NNNCATTGGT AGAAGCACA ATACATAGTC AAAACAGCAG
TCGTAOTTGG TTGTACTNN NNGTAACCA TCTTTCGTGT TATOTATCAG TTTTGTCTGC

1381 AGTATTAAAT AACAGAAAA TTTGCAAAAG GCAAGTAAAG AATATACATA TACTTAATTA
TCATAAATTA TTTGTCTTTT AAACGTTTC CGTTCATTTC TTATATGTAT ATGAATTAAT

1441 TACATAAAAT ATTGATACAG GAGGTAGAAA GAAATTAGT AAGCAGATAA TGGGGGCAAC
ATGTATTTA TAACTATGTC CTCCATCTTT CTTTAAATCA TTCGTCTATT ACCCCCCTTG

1501 AGAGTCCTCA GCAGAGCTTC CCTTCTAACA AAAGCAGCC CAATAAATTA TTTTTTTTTT
TCTCAGGAGT CGTCTCGAAG GGAAGATTGT TTTTCGTGCG GTTATTAAAT AAAAAAATAA

1561 CTAACAAAA GCAGCCTGAA AATCGAGCT GCAACATAA ATTAGCAATC GGCTGAAAGT

FIGURE 53E

GATTGTTTTT CGTCGGACTT TTTAGCTCGA CGTTTGTATC TAATCGTTAG CCGACTTTCA

 1621 GCGGGAGAAAT GCTGGCAGCT GTGCCAATAG TAAAGGGCTA CCTGGAGCCG GCGCGGTGGC
 CGCCCTCTTA CGACCGTCGA CACGGTTATC ATTTCCCGAT GGACCTCGGC CCGCGCACCG

 1681 TCACGCTGTA ATCCCAGCAC TTTGGGAGGG CGAGGCAACG CGGATCACCT GAGGTCGGGA
 AGTGGGACAT TAGGGTCGTG AAACCCICCC GTCGCGTTGC GCCTAGTGGA CTCCAGCCCT

 1741 GTTTGAGATC AGCCCGACCA ACATGGAGAA ACCCCGTCTC TACTAATAA AAAAATAA
 CAACTCTAG TCGGGCTGGT TGTACCTCTT TGGGGCAGAG ATGATTTTTT TTTTTTTTTT

 1801 AAAGGCATAA AATGAGCCGG GCATGGTGGC ACATGCCCTG CACATCCCAG CTGAGGCCAG
 TTTCCGTTTT TTA CTGCGCC CGTACCACCG TGTACGGAAC GTGTAGGTC GACTCCGTCC

 1861 AGAATTCAC TGAACCTGGG AGGTAGAGAT TCGGGTGAAG CGAGATCAG TCATTGCAC
 TCTTAAGTGA ACTTGGACCC TCCATCTCTA ACGCCACTTC GCTCTAGTGC AGTAACGTGA

 1921 CCAGCCTGGG CAAAAGAGC AAACCTTAGT CTCAAAAA AAANNCAA GAAAAA
 GGTCCGACCC CTTTTTCTCG TTTTGAATCA GAGTTTTTTT TTTTNNGTIT CTTTTT

FIGURE 54

Genomic Organization of PSM Gene

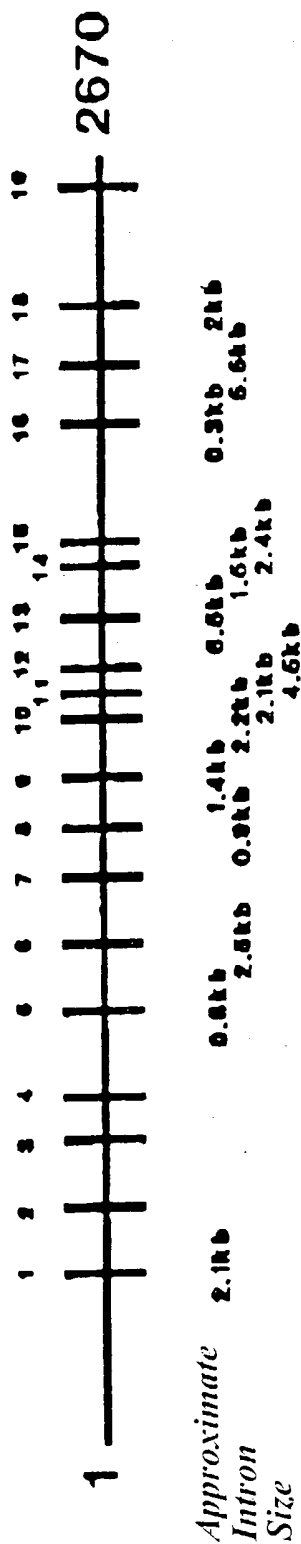


FIGURE 55A

10 20 30 40
 * * * * * *
 CTC AAA AGG GGC CGG ATT TCC TTC TCC TGG AGG CAG ATG TTG CCT CTC

50 60 70 80 90
 * * * * * * *
 TCT CTC GCT CGG ATT GGT TCA GTG CAC TCT AGA AAC ACT GCT GTG GTG

100 110 120 130 140
 * * * * * * *
 GAG AAA CTG GAC CCC AGG GTG GTT TAT AAA ATC CTC CAA TGA AGC TAC

150 160 170 180 190
 * * * * * * *
 TAA CAT TAC TCC AAA GCA TAA TAT GAA AGC ATT TTT GGA TGA ATT GAA

Met Lys Ala Phe Leu Asp Glu Leu Lys>

200 210 220 230 240
 * * * * * * *
 AGC TGA GAA CAT CAA GAA GTT CTT ATA TAA TTT TAC ACA GAT ACC ACA

Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile Pro His>

80/102

FIGURE 55B

250 260 270 280
* * * * *
TTT AGC AGG AAC AGA ACA AAA CTT TCA GCT TGC AAA GCA AAT TCA ATC

Leu Ala Gly Thr Glu Gln Asn Phe Gln Leu Ala Lys Gln Ile Gln Ser>

290 300 310 320 330
* * * * *
CCA GTG GAA AGA ATT TGG CCT GGA TTC TGT TGA GCT AGC ACA TTA TGA

Gln Trp Lys Glu Phe Gly Leu Asp Ser Val Glu Leu Ala His Tyr Asp>

340 350 360 370 380
* * * * *
TGT CCT GTT GTC CTA CCC AAA TAA GAC TCA TCC CAA CTA CAT CTC AAT

Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn Tyr Ile Ser Ile>

390 400 410 420 430
* * * * *
AAT TAA TGA AGA TGG AAA TGA GAT TTT CAA CAC ATC ATT ATT TGA ACC

Ile Asn Glu Asp Gly Asn Glu Ile Phe Asn Thr Ser Leu Phe Glu Pro>

440 450 460 470 480
* * * * *
ACC TCC TCC AGG ATA TGA AAA TGT TTC GGA TAT TGT ACC ACC TTT CAG

Pro Pro Pro Gly Tyr Glu Asn Val Ser Asp Ile Val Pro Pro Phe Ser>

81/102

FIGURE 55C

490 500 510 520
* * * * *
TGC TTT CTC TCC TCA AGG AAT GCC AGA GGG CGA TCT AGT GTA TGT TAA

Ala Phe Ser Pro Gln Gly Met Pro Glu Gly Asp Leu Val Tyr Val Asn>

530 540 550 560 570
* * * * *
CTA TGC ACG AAC TGA AGA CTT CTT TAA ATT GGA ACG GGA CAT GAA AAT

Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg Asp Met Lys Ile>

580 590 600 610 620
* * * * *
CAA TTG CTC TGG GAA AAT TGT AAT TGC CAG ATA TGG GAA AGT TTT CAG

Asn Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly Lys Val Phe Arg>

630 640 650 660 670
* * * * *
AGG AAA TAA GGT TAA AAA TGC CCA GCT GGC AGG GGC CAA AGG AGT CAT

Gly Asn Lys Val Lys Asn Ala Gln Leu Ala Gly Ala Lys Gly Val Ile>

680 690 700 710 720
* * * * *
TCT CTA CTC CGA CCC TGC TGA CTA CTT TGC TCC TGG GGT GAA GTC CTA

Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Ala Pro Gly Val Lys Ser Tyr>

82/102

FIGURE 55D

730 740 750 760
* * * * *
TCC AGA TGG TTG GAA TCT TCC TGG AGG TGG TGT CCA GCG TGG AAA TAT

Pro Asp Gly Trp Asn Leu Pro Gly Gly Gly Val Gln Arg Gly Asn Ile>

770 780 790 800 810
* * * * *
CCT AAA TCT GAA TGG TGC AGG AGA CCC TCT CAC ACC AGG TTA CCC AGC

Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro Gly Tyr Pro Ala>

820 830 840 850 860
* * * * *
AAA TGA ATA TGC TTA TAG GCG TGG AAT TGC AGA GGC TGT TGG TCT TCC

Asn Glu Tyr Ala Tyr Arg Arg Gly Ile Ala Glu Ala Val Gly Leu Pro>

870 880 890 900 910
* * * * *
AAG TAT TCC TGT TCA TCC AAT TGG ATA CTA TGA TGC ACA GAA GCT CCT

Ser Ile Pro Val His Pro Ile Gly Tyr Tyr Asp Ala Gln Lys Leu Leu>

920 930 940 950 960
* * * * *
AGA AAA AAT GGG TGG CTC AGC ACC ACC AGA TAG CAG CTG GAG AGG AAG

Glu Lys Met Gly Gly Ser Ala Pro Pro Asp Ser Ser Trp Arg Gly Ser>

FIGURE 55E

83/102

970 980 990 1000
 * * * * *
 TCT CAA AGT GCC CTA CAA TGT TGG ACC TGG CTT TAC TGG AAA CTT TTC

Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Thr Gly Asn Phe Ser>

1010 1020 1030 1040 1050
 * * * * *
 TAC ACA AAA AGT CAA GAT GCA CAT CCA CTC TAC CAA TGA AGT GAC AAG

Thr Gln Lys Val Lys Met His Ile His Ser Thr Asn Glu Val Thr Arg>

1060 1070 1080 1090 1100
 * * * * *
 AAT TTA CAA TGT GAT AGG TAC TCT CAG AGG AGC AGT GGA ACC AGA CAG

Ile Tyr Asn Val Ile Gly Thr Leu Arg Gly Ala Val Glu Pro Asp Arg>

1110 1120 1130 1140 1150
 * * * * *
 ATA TGT CAT TCT GGG AGG TCA CCG GGA CTC ATG GGT GTT TGG TGG TAT

Tyr Val Ile Leu Gly Gly His Arg Asp Ser Trp Val Phe Gly Gly Ile>

1160 1170 1180 1190 1200
 * * * * *
 TGA CCC TCA GAG TGG AGC AGC TGT TGT TCA TGA AAT TGT GAG GAG CTT

Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile Val Arg Ser Phe>

1210 1220 1230 1240
 * * * * *
 TGG AAC ACT GAA AAA GGA AGG GTG GAG ACC TAG AAG AAC AAT TTT GTT

Gly Thr Leu Lys Lys Glu Gly Trp Arg Pro Arg Arg Thr Ile Leu Phe>

84/102

FIGURE 55F

1250 1260 1270 1280 1290
* * * * * * *
TGC AAG CTG GGA TGC AGA AGA ATT TGG TCT TCT TGG TTC TAC TGA GTG

Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly Ser Thr Glu Trp>

1300 1310 1320 1330 1340
* * * * * * *
GGC AGA GGA GAA TTC AAG ACT CCT TCA AGA GCG TGG CGT GGC TTA TAT

Ala Glu Glu Asn Ser Arg Leu Leu Gln Glu Arg Gly Val Ala Tyr Ile>

1350 1360 1370 1380 1390
* * * * * * *
TAA TGC TGA CTC ATC TAT AGA AGG AAA CTA CAC TCT GAG AGT TGA TTG

Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu Arg Val Asp Cys>

1400 1410 1420 1430 1440
* * * * * * *
TAC ACC GCT GAT GTA CAG CTT GGT ACA CAA CCT AAC AAA AGA GCT GAA

Thr Pro Leu Met Tyr Ser Leu Val His Asn Leu Thr Lys Glu Leu Lys>

1450 1460 1470 1480
* * * * * * *
AAG CCC TGA TGA AGG CTT TGA AGG CAA ATC TCT TTA TGA AAG TTG GAC

Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr Glu Ser Trp Thr>

FIGURE 55G

1490 1500 1510 1520 1530
 * * * * * * *
 TAA AAA AAG TCC TTC CCC AGA GTT CAG TGG CAT GCC CAG GAT AAG CAA

Lys Lys Ser Pro Ser Pro Glu Phe Ser Gly Met Pro Arg Ile Ser Lys>

1540 1550 1560 1570 1580
 * * * * * * *
 ATT GGG ATC TGG AAA TGA TTT TGA GGT GTT CTT CCA ACG ACT TGG AAT

Leu Glv Ser Gly Asn Asp Phe Glu Val Phe Phe Gln Arg Leu Gly Ile>

1590 1600 1610 1620 1630
 * * * * * * *
 TGC TTC AGG CAG AGC ACG GTA TAC TAA AAA TTG GGA AAC AAA CAA ATT

Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Glu Thr Asn Lys Phe>

1640 1650 1660 1670 1680
 * * * * * * *
 CAG CGG CTA TCC ACT GTA TCA CAG TGT CTA TGA AAC ATA TGA GTT GGT

Ser Gly Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr Tyr Glu Leu Val>

1690 1700 1710 1720
 * * * * * * *
 GGA AAA GTT TTA TGA TCC AAT GTT TAA ATA TCA CCT CAC TGT GGC CCA

Glu Lys Phe Tyr Asp Pro Met Phe Lys Tyr His Leu Thr Val Ala Gln>

86/102

FIGURE 55H

1730 1740 1750 1760 1770
* * * * * * *
GGT TCG AGG AGG GAT GGT GTT TGA GCT AGC CAA TTC CAT AGT GCT CCC

Val Arg Gly Gly Met Val Phe Glu Leu Ala Asn Ser Ile Val Leu Pro>

1780 1790 1800 1810 1820
* * * * * * *
TTT TGA TTG TCG AGA TTA TGC TGT AGT TTT AAG AAA GTA TGC TGA CAA

Phe Asp Cys Arg Asp Tyr Ala Val Val Leu Arg Lys Tyr Ala Asp Lys>

1830 1840 1850 1860 1870
* * * * * * *
AAT CTA CAG TAT TTC TAT GAA ACA TCC ACA GGA AAT GAA GAC ATA CAG

Ile Tyr Ser Ile Ser Met Lys His Pro Gln Glu Met Lys Thr Tyr Ser>

1880 1890 1900 1910 1920
* * * * * * *
TGT ATC ATT TGA TTC ACT TTT TTC TGC AGT AAA GAA TTT TAC AGA AAT

Val Ser Phe Asp Ser Leu Phe Ser Ala Val Lys Asn Phe Thr Glu Ile>

1930 1940 1950 1960
* * * * * * *
TGC TTC CAA GTT CAG TGA GAG ACT CCA GGA CTT TGA CAA AAG CAA CCC

Ala Ser Lys Phe Ser Glu Arg Leu Gln Asp Phe Asp Lys Ser Asn Pro>

FIGURE 55I

1970 1980 1990 2000 2010
 * * * * * * *
 AAT AGT ATT AAG AAT GAT GAA TGA TCA ACT CAT GTT TCT GGA AAG AGC

Ile Val Leu Arg Met Met Asn Asp Gln Leu Met Phe Leu Glu Arg Ala>

2020 2030 2040 2050 2060
 * * * * * * *
 ATT TAT TGA TCC ATT AGG GTT ACC AGA CAG GCC TTT TTA TAG GCA TGT

Phe Ile Asp Pro Leu Gly Leu Pro Asp Arg Pro Phe Tyr Arg His Val>

2070 2080 2090 2100 2110
 * * * * * * *
 CAT CTA TGC TCC AAG CAG CCA CAA CAA GTA TGC AGG GGA GTC ATT CCC

Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly Glu Ser Phe Pro>

2120 2130 2140 2150 2160
 * * * * * * *
 AGG AAT TTA TGA TGC TCT GTT TGA TAT TGA AAG CAA AGT GGA CCC TTC

Gly Ile Tyr Asp Ala Leu Phe Asp Ile Glu Ser Lys Val Asp Pro Ser>

2170 2180 2190 2200
 * * * * * * *
 CAA GGC CTG GGG AGA AGT GAA GAG ACA GAT TTA TGT TGC AGC CTT CAC

Lys Ala Trp Gly Glu Val Lys Arg Gln Ile Tyr Val Ala Ala Phe Thr>

2210 2220 2230 2240 2250
 * * * * * * *
 AGT GCA GGC AGC TGC AGA GAC TTT GAG TGA AGT AGC CTA AGA GGA TTC

Val Gln Ala Ala Ala Glu Thr Leu Ser Glu Val Ala

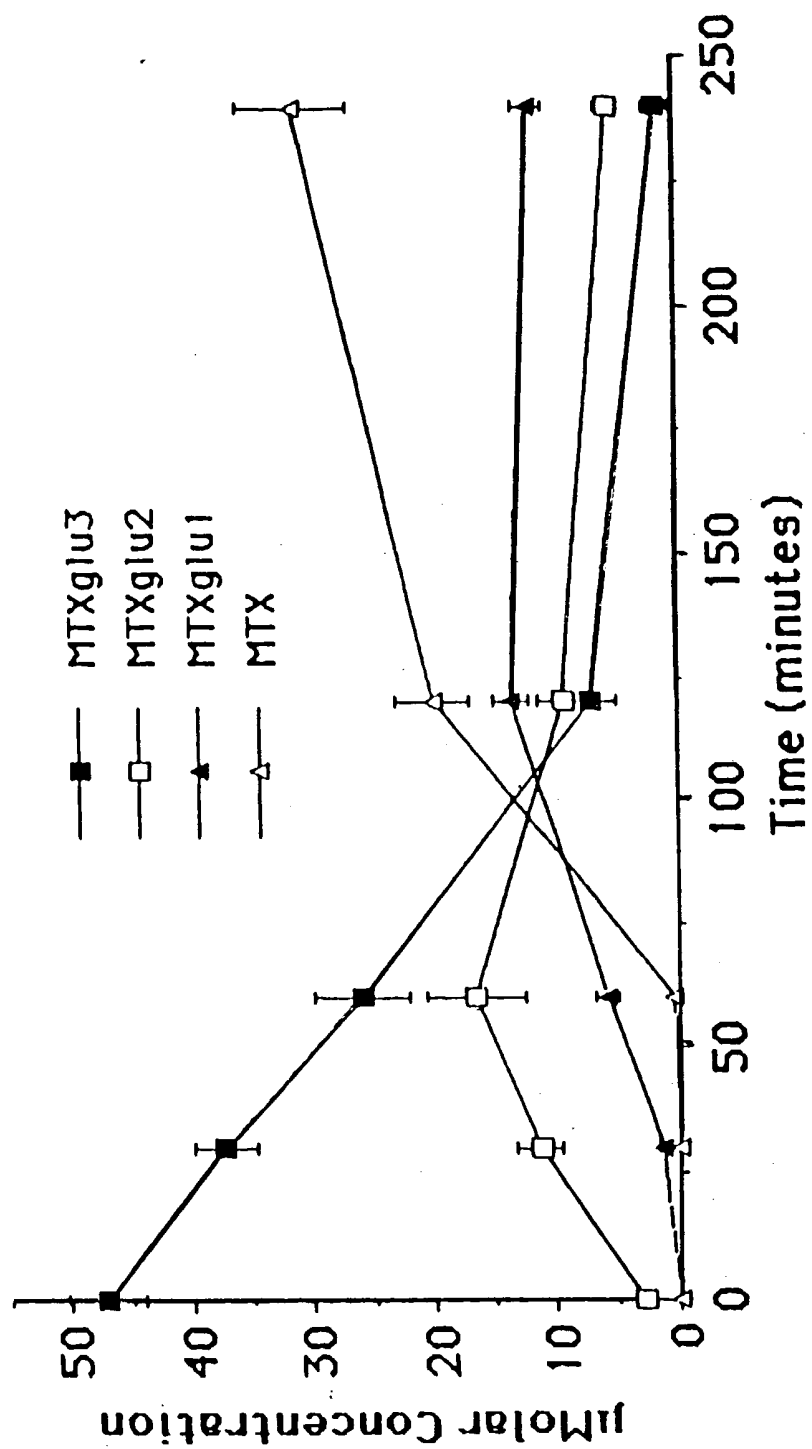
FIGURE 53J

2260 2270 2280 2290 2300
* * * * * *
TTT AGA GAA TCC GTA TTG AAT TTG TGT GGT ATG TCA CTC AGA AAG AAT

2310 2320 2330 2340 2350
* * * * * *
CGT AAT GGG TAT ATT GAT AAA TTT TAA AAT TGG TAT ATT TGA AAT AAA

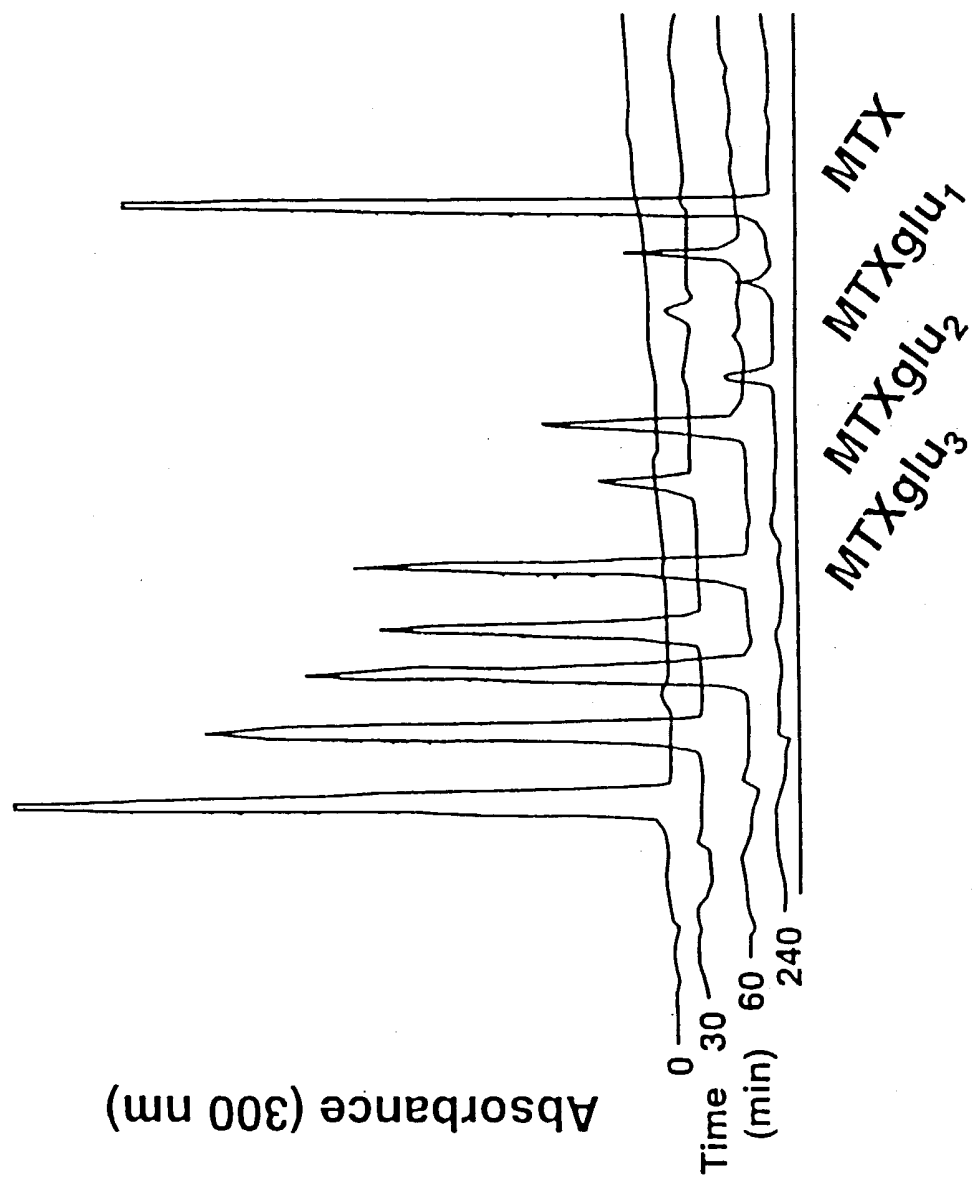
2360 2370 2380
* * * * *
GTT GAA TAT TAT ATA TAA AAA AAA AAA AAA AAA AA

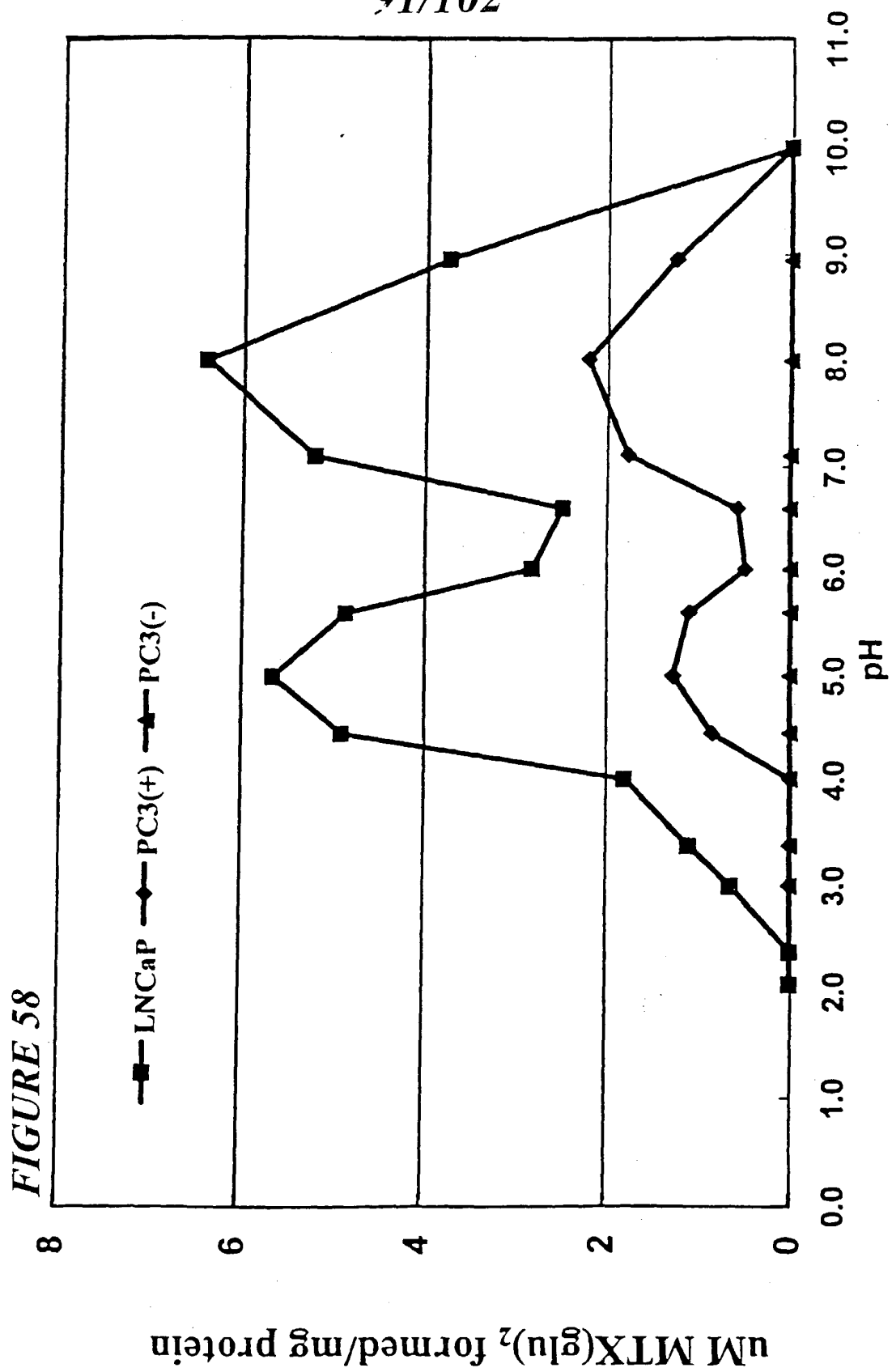
FIGURE 56

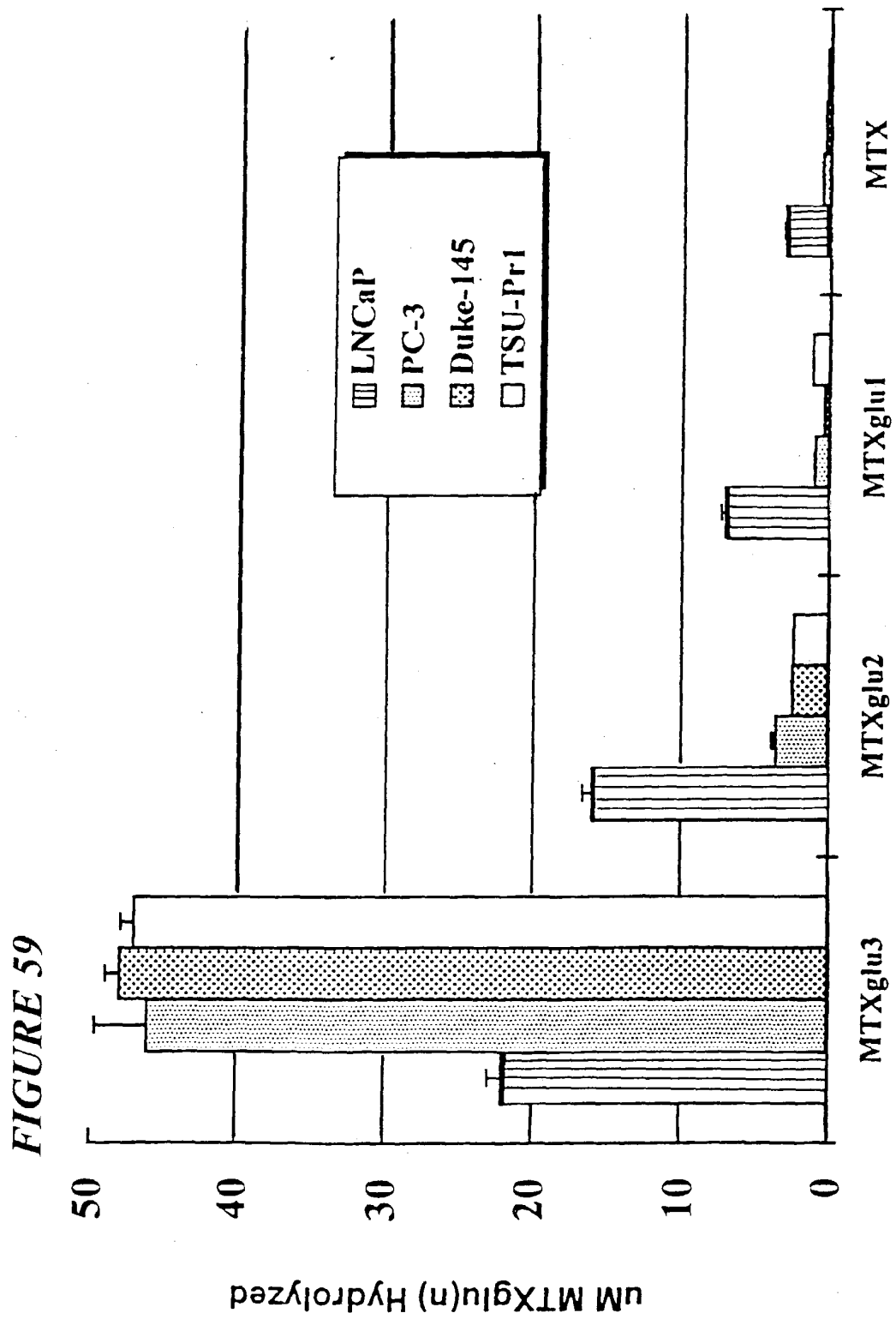


90/102

FIGURE 57







93/102

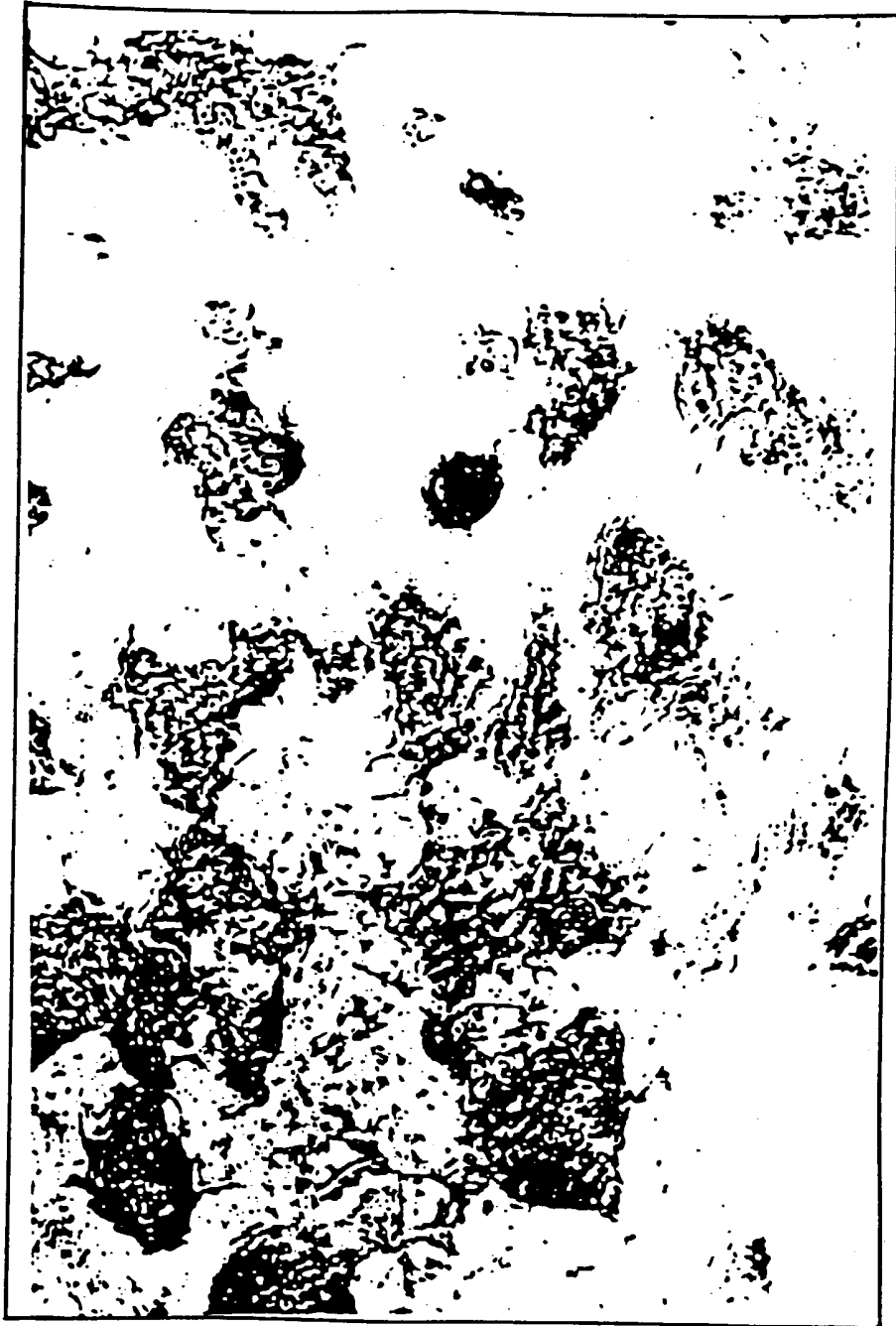


FIGURE 60A

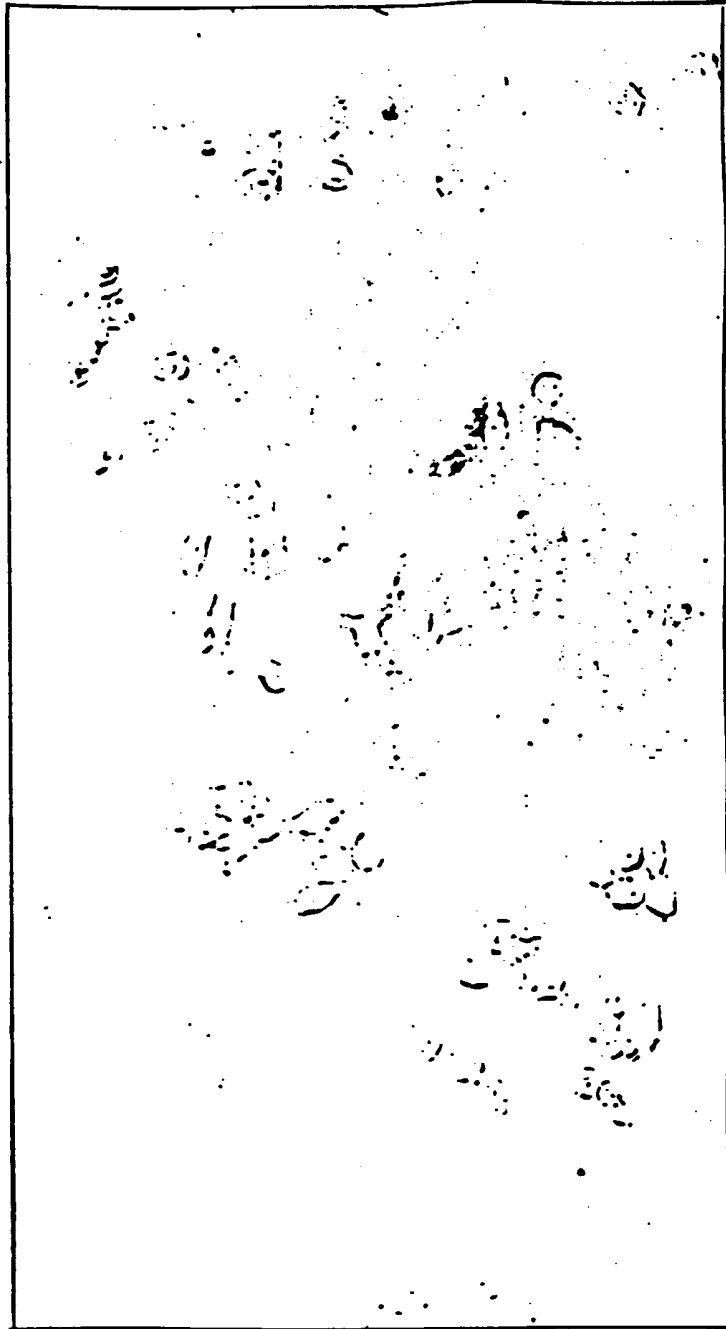
94/102

FIGURE 60B



95/102

FIGURE 60C



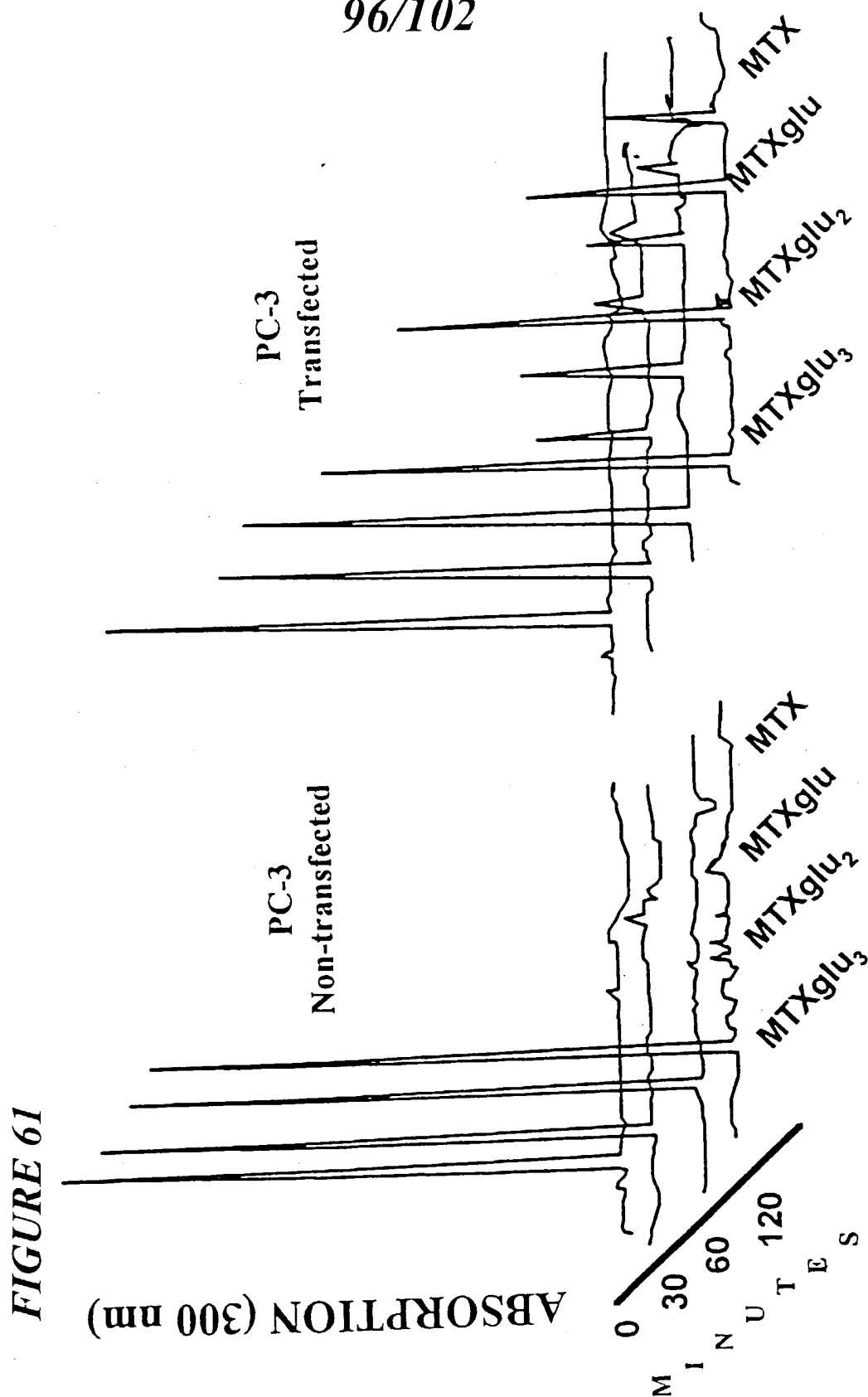


FIGURE 62

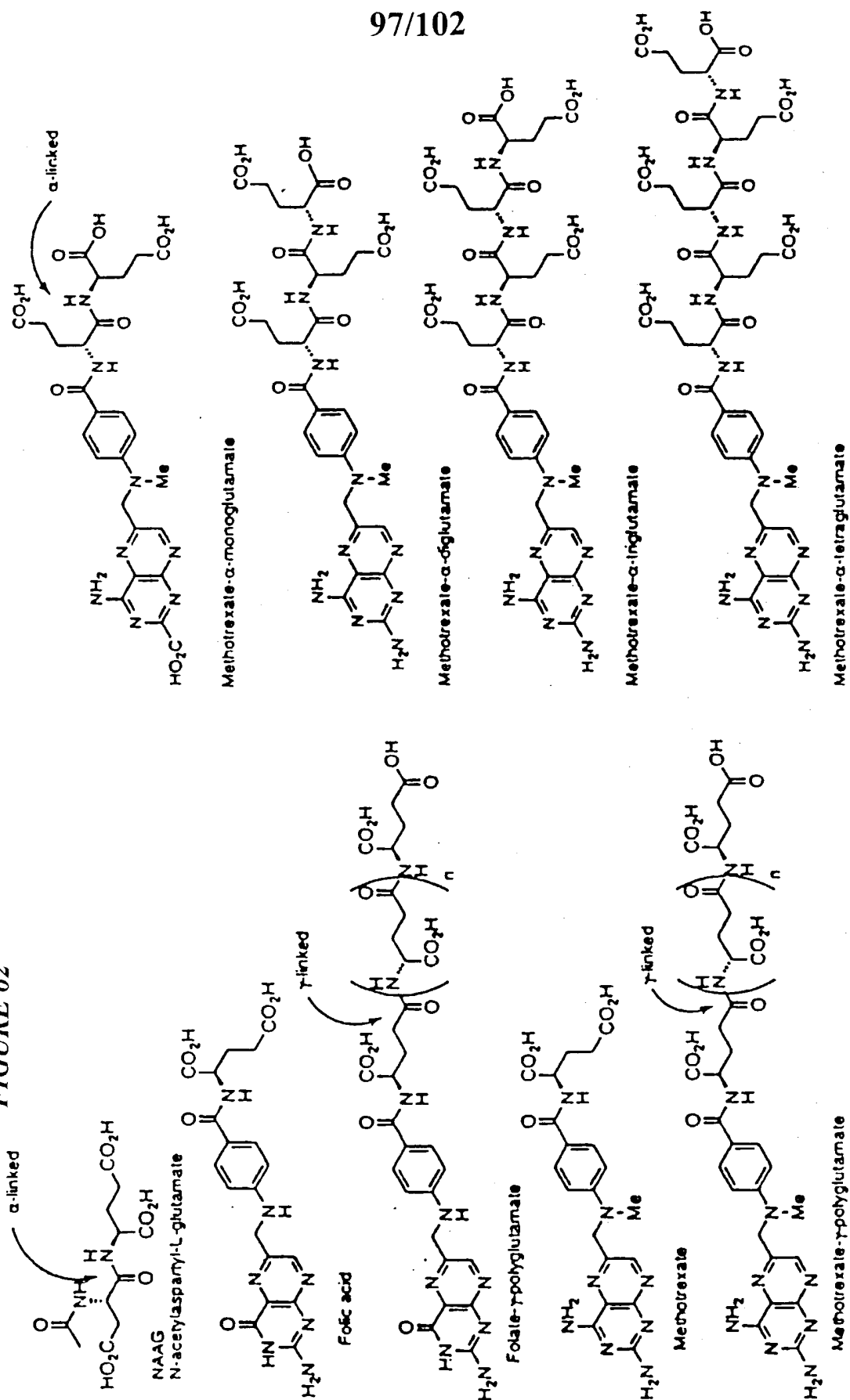
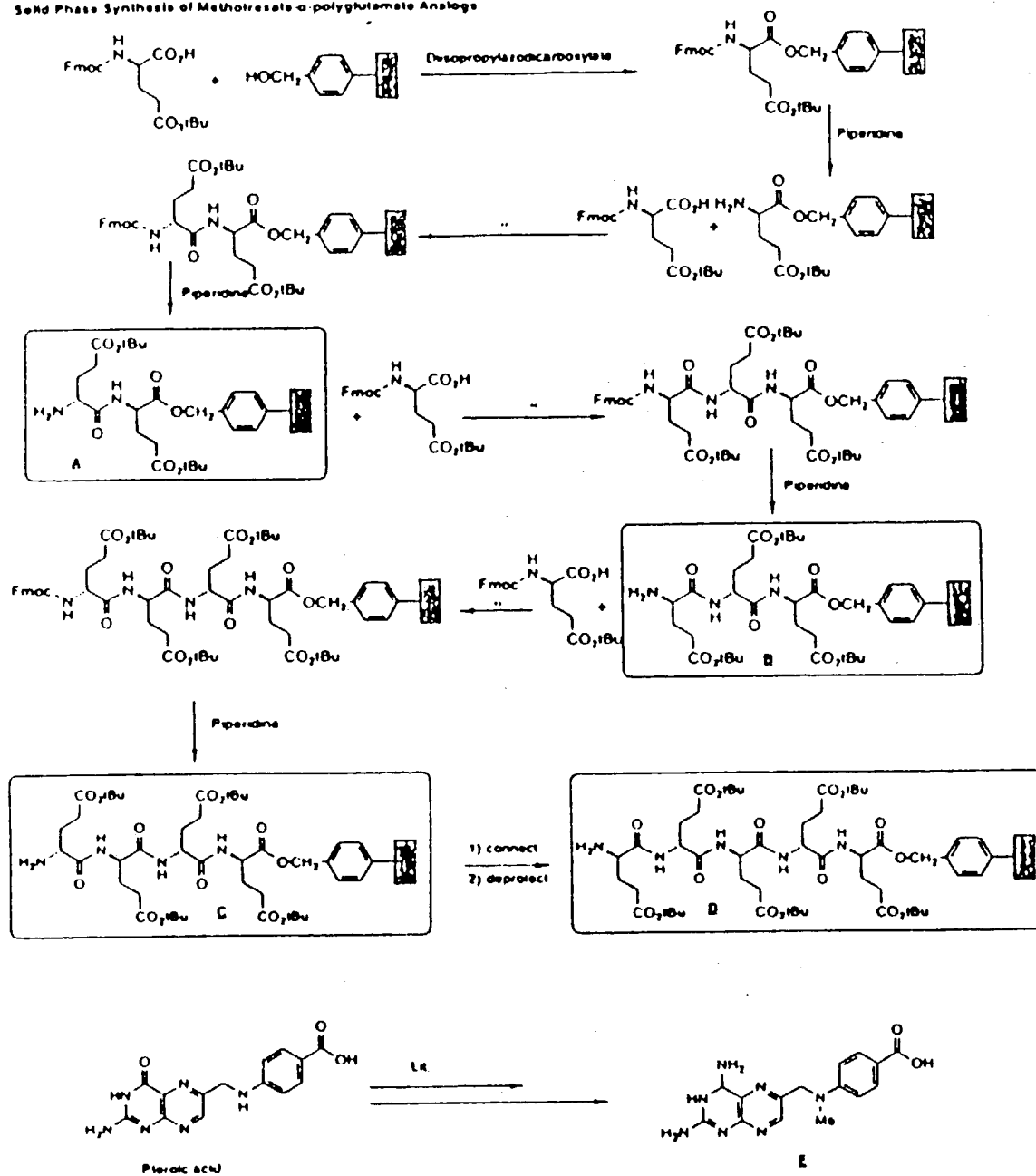


FIGURE 63A

Solid Phase Synthesis of Methotresate α -polyglutamate Analogs



99/102

FIGURE 63B

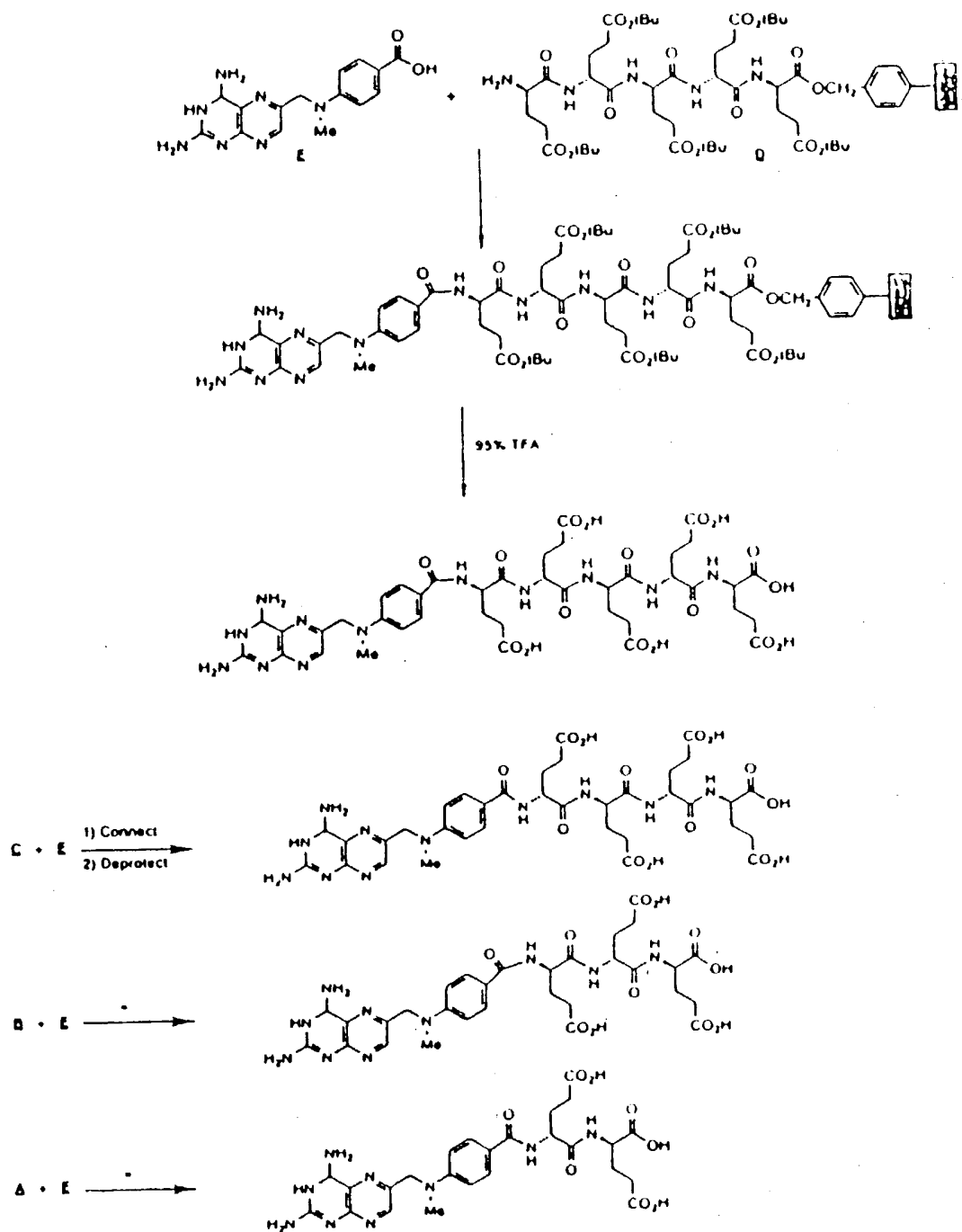


FIGURE 64

Sequence Analysis of microsatellite instability in PSM gene

<u>Sample</u>	<u>Sequence</u>	<u>PSM EXPRESSION (IMMUNO STAIN)</u>
Genomic	T ₉ GC(TTTTG) ₈ (TTTG) ₃ T ₇	
LNCaP	T ₉ GC(TTTTG) ₆ (TTTG) ₃ T ₇	positive
PC-3	T ₉ GC(TTTTG) ₈ (TTTG) ₃ T ₆	negative
DU145	T ₁₀ GC(TTTTG) ₅ (TTTG) ₂ T ₇	negative
T4 (tumor)	T ₁₀ GC(TTTTG) ₆ (TTTG) ₃ T ₇	positive
N4(paired normal)	T ₉ GC(TTTTG) ₆ (TTTG) ₃ T ₇	positive

FIGURE 65

Genomic Organization of PSM Gene

101/102

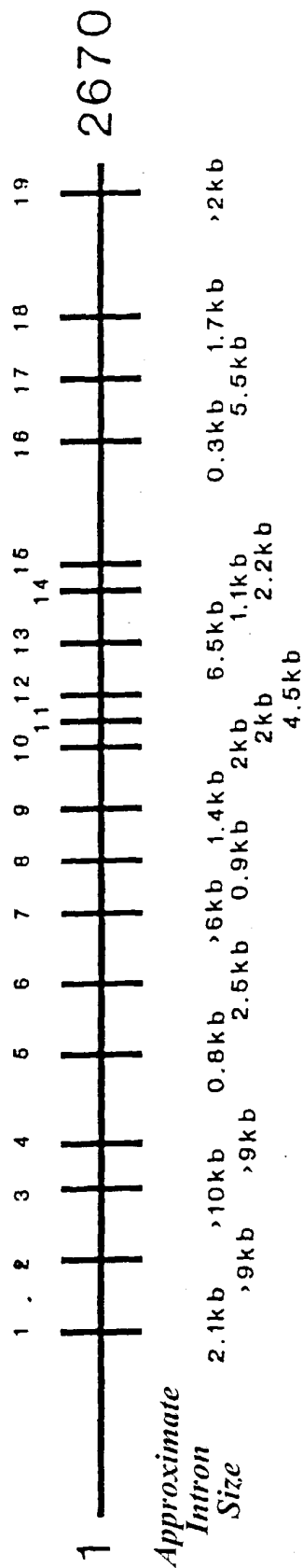


FIGURE 66
Location of microsatellite in PSM Gene

